Role of the microenvironment in maintenance and resistance of leukemic stem cells in Chronic Myelogenous Leukemia. BMP pathway and mechanical forces

Bastien Laperrousaz

THESE

en vue de l'obtention du grade de
Docteur de l'Université de Lyon, délivré par l'École Normale Supérieure de Lyon
Discipline : ***Sciences de la vie***

Laboratoire de physique de l'ENS de Lyon
Centre de Recherche en Cancérologie de Lyon
École Doctorale Biologie Moléculaire Intégrative et Cellulaire

présentée et soutenue publiquement le 30 mars 2015 par

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List of abbreviations

ABL :	Abelson
ACTR :	Activin Receptor
AFM :	Atomic Force Microscopy
ALL :	Acute Lymphoblastic Leukemia
AML :	Acute Myeloid Leukemia
AP :	Acceleration Phase
BAMBI :	BMP and Activin Bound Inhibitor
BC :	Blast crisis
BCR :	Breakpoint Cluster Region
BCR-ABL :	Breakpoint cluster region-Abelson
BGUS :	Beta-glucuronidase
BIT :	BSA, Insulin, Transferrin
BM :	Bone Marrow
BMI-1 :	B lymphoma Mo-MLV Insertion region 1 homolog
BMP :	Bone Morphogenetic Protein
BMPR :	Bone Morphogenetic Protein Receptor
BMT :	Bone Marrow Transplantation
BSA :	Bovine Serum Albumin
CCyR :	Complete Cytogenetic Remission
CD :	Cluster of Differentiation
CFC :	Colony Forming Cell
CFU :	Colony Forming Unit
CFU-Baso :	Colony Forming Unit-Basophile
CFU-E :	Colony Forming Unit-Erythroid
CFU-Eo :	Colony Forming Unit-Eosinophile
CFU-GM :	Colony Forming Unit-Granulocyte, Macrophage
CFU-Mix :	Colony Forming Unit-Mix
CFU-MK :	Colony Forming Unit – Megakaryocyte
CHR :	Complete Hematologic Response
CLL :	Chronic Lymphocytic Leukemia
CML :	Chronic Myelogenous Leukemia
CMR :	Complete Molecular Response
CP :	Chronic Phase
CR :	Cytogenetic Remission
CSC :	Cancer Stem Cell
DMEM :	Dulbecco's Modified Eagle's Medium
DNA :	Deoxyribonucleic Acid
ECM :	Extracellular Matrix
EDTA :	Ethylenediaminetetraacetic acid
EPO :	Erythropoietin
FACS :	Fluorescence Activated Cell Sorting
FAK :	Focal Adhesion Kinase
FCS :	Fetal Calf Serum
FISH :	Fluorescence In Situ Hybridization
FITC :	Fluorescein isothiocyanate
FLRG :	Follistatin Related Gene
GFP :	Green Fluorescent Protein
G-CSF :	Granulocyte-Colony Stimulating Factor
GM-CSF :	Granulocyte Macrophage-Colony Stimulating Factor
HPRT :	Hypoxanthine-guanine-Phospho-Ribosyl-Transferase

HR :	Hematologic Response
HSC :	Hematopoietic Stem Cell
ID :	Inhibitor of Differentiation
IFN :	Interferon
IL :	Interleukin
IMDM :	Iscove's Modified Dulbecco's Medium
kDa :	kilo Dalton
LIC :	Leukemia Initiating Cell
LIF :	Leukemia Inhibitory Factor
LIN :	Lineage
LTC-IC :	Long Term Culture-Initiating Cell
LT-HSC :	Long Term-Hematopoietic Stem Cell
LSC :	Leukemic Stem Cell
MNC :	MonoNucleated Cell
MR :	Molecular Response
MSC :	Mesenchymal Stromal Cell
NOD/SCID :	Non-Obese Diabetic -Severe Combined Immune-Deficient
OPL :	Optical Path Lenght
PBS :	Phosphate Buffered Saline
PDMS :	Polydiméthylsiloxane
PE :	Phycoerythrin
PE-Cya5 :	Phycoerythrin Cyanine 5
Ph :	Philadelphia chromosome
qRT-PCR :	quantitative Reverse Transcription - Polymerase Chain Reaction
QPI :	Quantitative Phase Imaging
QPM :	Quantitative Phase Microscopy
RNA :	Ribonucleic acid
RGM :	Repulsive Guidance Molecule
RPMI :	Roswell Park Memorial Institute
RUNX :	Runt related
SC :	Stem Cell
SCF :	Stem Cell Factor
SCT :	Stem Cell Transplantation
SMAD :	Small body size mothers against decapentaplegic
ST-HSC :	Short Term-Hematopoietic Stem Cell
TBP :	TATA box Binding Protein
TGF-β :	Tumor Growth Factor-β
TIFP :	Tumor Interstitial Fluid Pressure
TKI :	Tyrosine Kinase Inhibitor
TPO :	Thrombopoïetin
VLA :	Very Late Antigen
α-MEM :	Minimum Essentiel Medium Alpha

Introduction

Chapter 1 : Physiology and pathology of the hematopoietic system

I. Hematopoiesis

Hematopoiesis is a physiological process resulting in the production and perpetual replacement of various blood elements. Human hematopoietic tissue is highly prolific, as about 10^{13} blood cells are produced every day in adult man under normal physiological conditions.[1] This permanent production activity is provided by a small cell fraction located in bone marrow called hematopoietic stem cell (HSC). Hence, all blood cells are derived from a common pluripotent HSC capable of both self-renewal and further differentiation into committed cells. Hematopoiesis is a complex and tightly regulated cellular system that allows fine adjustments to blood cell production to fit with the needs of the organism in physiological conditions or in response to external aggressions (bleeding, diseases...).

In the embryo, hematopoiesis begins within the yolk sac and then gradually migrates to the liver and spleen in the early stages of human embryonic development. It only becomes medullar after the 15th week.[2] In the fetus, hematopoietic activity is found in the liver, spleen, thymus, lymph nodes and bone marrow. In human adults, hematopoiesis is limited to short and flat bones like the hip bone, pelvis, sternum, ribs or vertebrae while in rodents, spleen also keep a hematopoietic activity during adulthood. However, maturation, activation, and in some cases proliferation of lymphoid cells occur in secondary lymphoid organs (spleen, thymus and lymph nodes). In adults, hematopoiesis can sometimes also occur outside the bone marrow in pathological processes, it is then referred to as extramedullary hematopoiesis. For example, it can be caused by myelofibrosis, after fibrotic changes within the bone marrow "crowd out" hematopoietic cells, causing them to migrate to other sites such as the liver and spleen.[3]

II. Hematopoietic compartments

In the hematopoietic system, four cellular compartments are generally defined: hematopoietic stem cells (HSCs), progenitors, precursors and mature cells.[1] These cells undergo sequential divisions as they mature and mature cells have limited life spans. HSCs are able to produce multipotent progenitors (MPPs), that can in turn differentiate into common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs), thus giving rise to myeloid or lymphoid lineage, respectively. Progenitors will further proliferate and differentiate into precursors, finally leading to the production of mature cells. HSCs, progenitors and precursors are localized in the bone marrow whereas mature cells circulate in the peripheral blood.

Mature cells provide specialized functions and have a limited lifespan. They are easily identifiable on the basis of morphological criteria, phenotypic markers but also functional properties. In the myeloid hematopoietic lineage, erythrocytes, platelets and different white blood cell types, such as monocytes and granulocytes are formed. In the lymphoid lineage, T cells, B cells and NK cells are generated. The hierarchical model of hematopoiesis is shown in Figure 1.

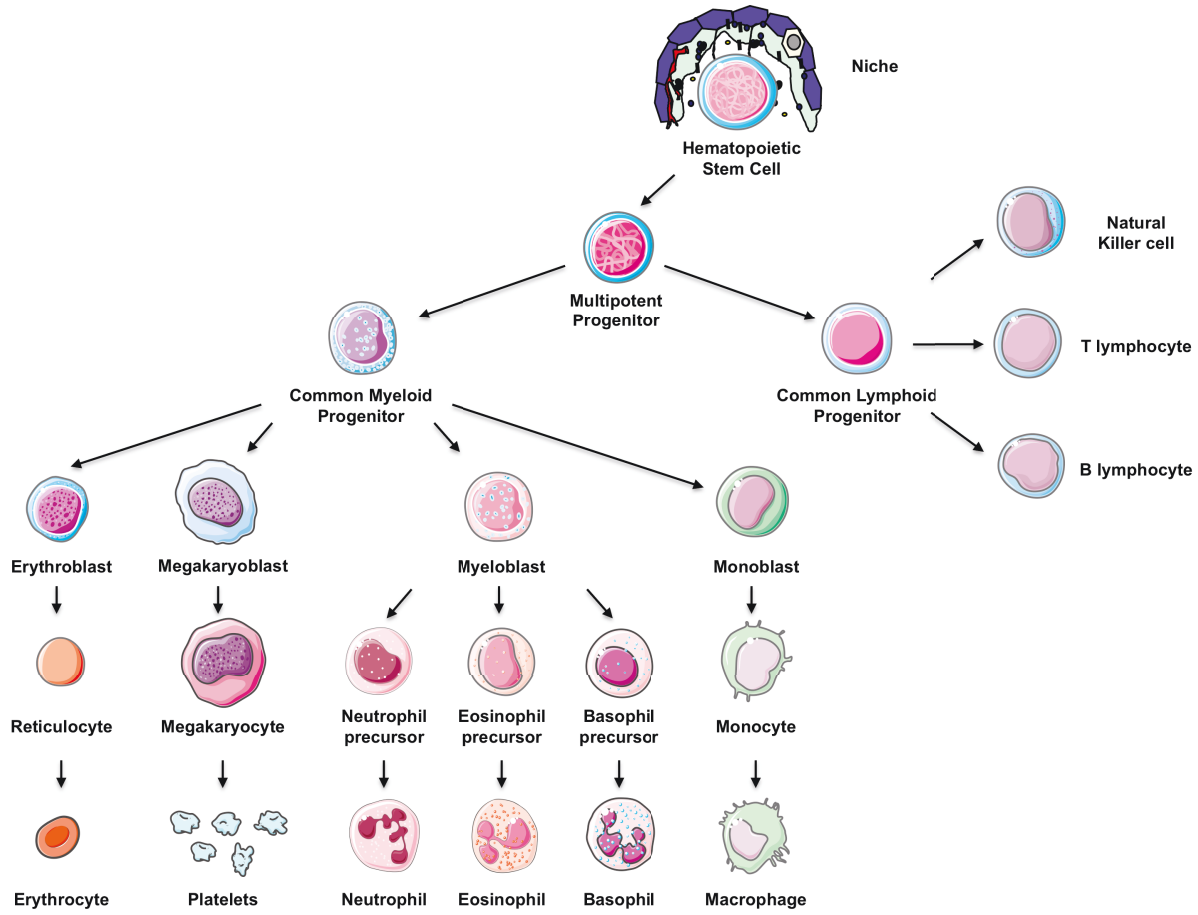

Figure 1 : From hematopoietic stem cells to mature and functional hematopoietic cells. Diagram showing the development of the different blood cells from hematopoietic stem cell to mature cells. HSC gives rise to multipotent progenitors that in turn will give rise to common myeloid progenitors (CMP) and common lymphoid progenitors (CLP). CLP progeny include B cells, T cells and NK cells while CMP generates different types of precursors that will eventually give rise to erythrocytes, platelets, neutrophils, eosinophils, basophils and macrophages.

Erythrocytes are primarily involved in gas exchange between the organism and the external environment and represent more than 95% of the total blood cell population. The cytoplasm of erythrocytes is rich in hemoglobin, an iron-containing protein that can bind oxygen and is responsible for the red color of the cells. Erythrocytes are flexible and oval biconcave disks ranging from 6 µm to 8 µm of diameter, lacking cell nucleus and most

organelles, in order to accommodate maximum space for hemoglobin. They possess a half-life of about 120 days. Leukocytes (neutrophils, eosinophils, basophils, monocytes and lymphocytes) are major players in the immune system. They represent only a small proportion of the total blood cell population (less than 0.1%) and have a very variable lifetime ranging from 8h for some polynuclear cells to up to several months for some lymphocytes. Finally, the platelets also called thrombocytes are mainly involved in the clotting process and account for about 3% of the total blood cell population. Their half-life ranges from about 7 days to 10 days. Platelets have no nucleus: they are fragments of cytoplasm that are derived from the megakaryocytes of the bone marrow, and then enter the peripheral blood circulation. Platelets are biconvex discoid structures shaped like a lens ranging from 2 µm to 3 µm of diameter and only found in mammals.[4]

Precursors are derived from the differentiation of the progenitors. They have lost all self-renewal potential, are engaged towards differentiation in a unique hematopoietic lineage and are fated to proliferation and cell maturation. The precursors do not generate colonies in semi-solid medium (CFC assay), but are morphologically identifiable (microscopic observations, staining...). Each precursor type will undergo amplification in cell number (3 to 5 cell divisions), with morphological criteria specific to each stage of maturation, corresponding to the gradual acquisition of specific components of terminally differentiated cells, such as hemoglobin in erythrocytes (oxygen transport) or granules in neutrophils (phagocytosis). Precursors ultimately give rise to mature and functional cells released into the bloodstream.

Progenitors are derived from HSCs that are committed to differentiation towards myeloid or lymphoid lineages. Unlike HSCs, they are no longer able to self-renew but have acquired a strong proliferative capability while remaining able to differentiate. However, self-renewal was recently observed in differentiated macrophage populations, suggesting that it may not be a specific HSC attribute.[5] Like HSCs, progenitors cannot be morphologically identifiable, but can be detected by functional testing. The CFC assay (Colony Forming Cell) allows the identification of multipotent and committed myeloid progenitors. Inspired from previous work,[6] this test is based on their ability to form colonies in a semi-solid medium containing methylcellulose, supplemented with cytokines and growth factors (Methods). A CFC colony will be obtained after 2 weeks of culture from the proliferation and the differentiation of a single hematopoietic progenitor. Different types of myeloid colonies can be observed and identified under a microscope: CFU-Mix (colony forming unit-granulocyte, erythroids, macrophages, megakaryocytes), CFU-GM (colony forming unit-granulocyte, macrophages), CFU-E (colony forming unit-Erythroids), CFU-Eo (Colony Forming Unit-Eosinophils) and CFU-Baso (Colony Forming Unit-Basophils). The size of a given colony informs about the degree of maturity of the progenitor at the origin of the colony, the most

immature progenitors having a higher proliferation potential in methylcellulose compared to more mature progenitors. The most immature colonies are characterized by "early CFU" and more mature colonies "late CFU." However, megakaryocytic progenitors cannot be detected by the CFC assay described above because of the presence of TGF-β in the semi-solid medium used, which inhibits their growth. To circumvent this problem, the CFU-MK assay (Colony Forming Unit - megakaryocytes) was developed from a semi-solid medium containing collagen supplemented with horse serum. Moreover, human lymphoid progenitors seem to require stromal support for growth and therefore cannot be detected by CFC assay.

Since most mature blood cells are terminally differentiated and short-lived in blood circulation, it is essential to generate functional blood cells to maintain the hematopoietic system in a steady state through the proliferation and differentiation of HSCs. In adults, HSCs are principally located in the bone marrow and represent less than 0.01% of the total bone marrow cell population. They can also be found in umbilical cord blood and, in small numbers, in peripheral blood. With regard to morphology, hematopoietic stem cells resemble lymphocytes. They are round cells with a rounded nucleus and low cytoplasm-to-nucleus ratio. HSCs have an extensive self-renewal and proliferative potential as well as the capability to differentiate into progenitors of all blood cell lineages. Self-renewal refers to the ability to produce daughter cells with identical characteristics and is necessary to ensure that the HSC pool is sustained throughout adult life. The balance between self-renewal and differentiation is regulated to maintain the HSC pool while generating sufficient progeny. This can be achieved by asymmetric cell division where one HSC gives rise to an identical daughter cell and a differentiated progeny (Figure 2). In addition, HSC can also divide symmetrically to generate two identical daughter cells or two differentiated progenies (Figure 2). This self-renewal ensures that the HSC pool is protected from exhaustion. However, the frequency of these cell divisions varies amongst the HSCs. In this respect, the most primitive HSCs only rarely divide, whereas other HSCs divide more frequently. The formation of mature and functional blood cells by the HSCs occurs via several consecutive cell divisions and maturation stages. The initial asymmetrical cell division is followed by several symmetrical cell divisions, during which two identical daughter cells are generated. These newly formed cells are more differentiated than their parental cells and become increasingly committed to either the myeloid or the lymphoid lineage.

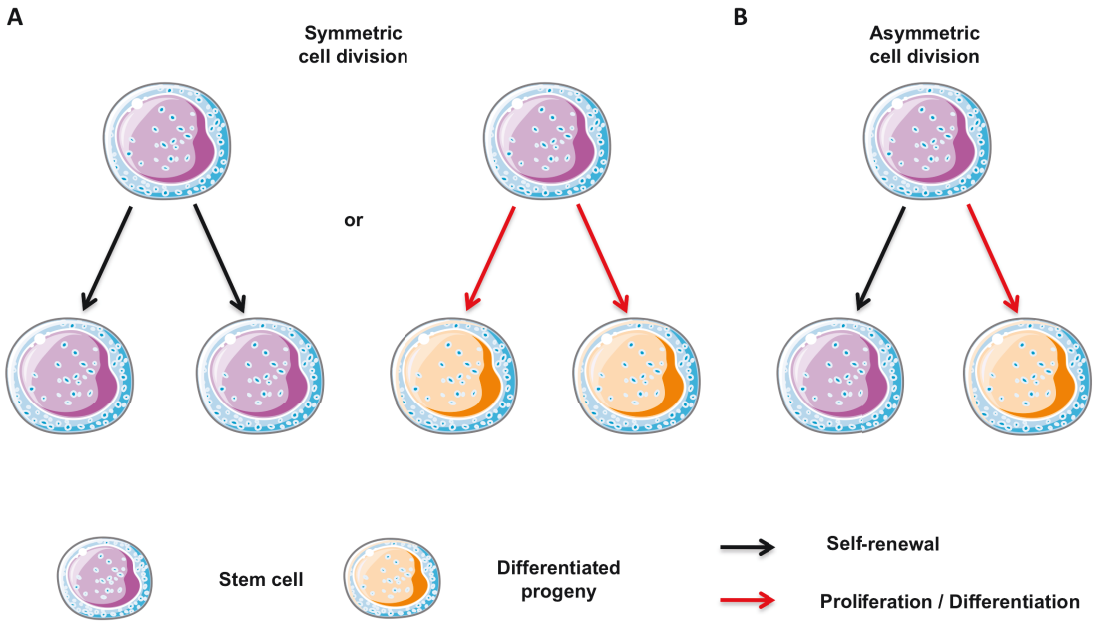

Figure 2 : Stem cell self-renewal, proliferation and differentiation. Stem cells are able to divide through symmetric or asymmetric divisions. (A) Symmetric division produces two identical daughter cells that can either be identical to the parent cell or different, with a more restricted potential compared to the parent cell. The production of two SC identical to the parent SC allows the maintenance of the SC population, while the production of two engaged daughter cells will lead to their progressive proliferation and differentiation until the formation of terminally differentiated and functional cells. (B) Asymmetric division leads to the formation of a daughter cell identical to the parent cell and of a second cell with more restricted potential.

III. Hematopoietic Stem Cells

Continuous production of mature blood cells is allowed by the existence of a small cell fraction in the bone marrow called hematopoietic stem cell (HSC). The existence of a multipotent HSC has first been proved in 1961.[7] It has been demonstrated that irradiation of mice destroys their hematopoietic tissue and induces a fatal medullar aplasia in the absence bone marrow transplantation. On the contrary, if a transplant is performed after irradiation (injection of bone marrow from syngenic mice) the animals do not die of myelosuppression. Ten days after transplantation, the spleen of the recipient mice were filled with macroscopic cell clusters. These colonies called “Colony Forming Unit-Spleen” (CFU-S) were mixed colonies, as they were constituted of cells belonging to different lineages. This experiment was repeated after introduction of various chromosomal abnormalities in the transplanted cells. It was then observed that all the cells of a unique colony displayed the same chromosomal abnormality, thus demonstrating that all cells of a colony originated from the same multipotent hematopoietic stem cell.[7] Finally, the self-renewal capability of these cells

has also been evidenced as the injection of a splenic colony into a new irradiated mouse allowed the reconstitution of hematopoiesis in this mouse

HSCs are mostly found in the bone marrow where they still represent a small cell population. Their presence in the blood is not excluded but they remain extremely rare under normal physiological conditions. It is estimated that one out of 10^4 bone marrow cells could be a HSC against one out of 10^5 in the circulating blood. HSC can be obtained by bone marrow aspiration (usually from the pelvis, at the iliac crest) for stem cell transplantations (SCT). The cells can be removed as liquid to perform a smear and to look at their morphology or they can be removed via core biopsy to maintain the bone marrow architecture. In order to obtain HSCs from the circulating peripheral blood, donors are injected with a cytokine, such as granulocyte-colony stimulating factor (G-CSF), that induces the detachment of HSC from their bone marrow stroma and their circulation in the blood vessels where they can easily be harvested. However, the percentage of HSCs among bone marrow cells is still very low. Thus, it is very important to be able to identify these cells to characterize their functional properties.

HSC function is usually assessed in-vitro by long-term culture initiating-cell (LTC-IC)[8,9] assay and *in-vivo* by bone marrow engraftment of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice.[10] LTC-IC assays detect primitive progenitors that are able to form CFC colonies after 5 weeks culture on a stromal feeder layer (Methods).[8] NOD/SCID-repopulating cells are capable of reconstituting lethally irradiated NOD/SCID mice with a singe cell and are more primitive than most LTC-IC.[10] Based on their ability to reconstitute the hematopoietic system of lethally irradiated mice, mouse HSCs can be separated into three distinct groups: long-term HSCs (LT-HSCs) which are able to generate all types of blood cells for the lifetime of the animals and reconstitute secondary hosts; short-term HSCs (ST-HSCs) which derive from LT-HSCs but can only reconstitute myeloid and/or lymphoid lineages for a limited period of time and multipotent progenitors (MPPs), which are generated by ST-HSCs, have no detectable self-renewal capability and only transiently reconstitute lethally irradiated mice (Figure 3). HSCs are also characterized by their ability to reject some vital dyes such as Hoechst 33342 or Rhodamine 123 via the presence of efflux pumps on their surface.[11] The expression of these transporters such as MDR1 (Multi-drug resistance 1) and ABCG2 (ATP-binding cassette subfamily G member 2) is correlated with the immature status of hematopoietic cells.[12] In addition, it has been suggested that the frequency of HSCs division is smaller compared to hematopoietic progenitors and precursors.[13,14] This property can be demonstrated by conventional methods such as cell-cycle analysis, flow cytometry or immunohistochemistry but also by cell staining with BrdU (bromodeoxyuridine) or GFP (Green Fluorescent Protein), thus allowing a spatial and temporal monitoring of HSCs *in-vivo*. During their successive divisions, cells gradually lose

their staining, until the signal is no longer detectable. In contrast, cells that have completed few divisions retain their staining and are easily detectable. Finally, *in-vivo* experiments helped to highlight the existence of two different HSCs subpopulations: a population of cells called "dormant" or quiescent (about 30%) that divide every 145 to 193 days and a population of more active cells (about 70%) which divide every 28 to 36 days.[15,16] While the two subpopulations are capable of reconstituting normal hematopoiesis after transplantation in a first previously irradiated animal, only the population of quiescent cells possesses long-term recovery capabilities as demonstrated by serial transplantation assays.[15,16]

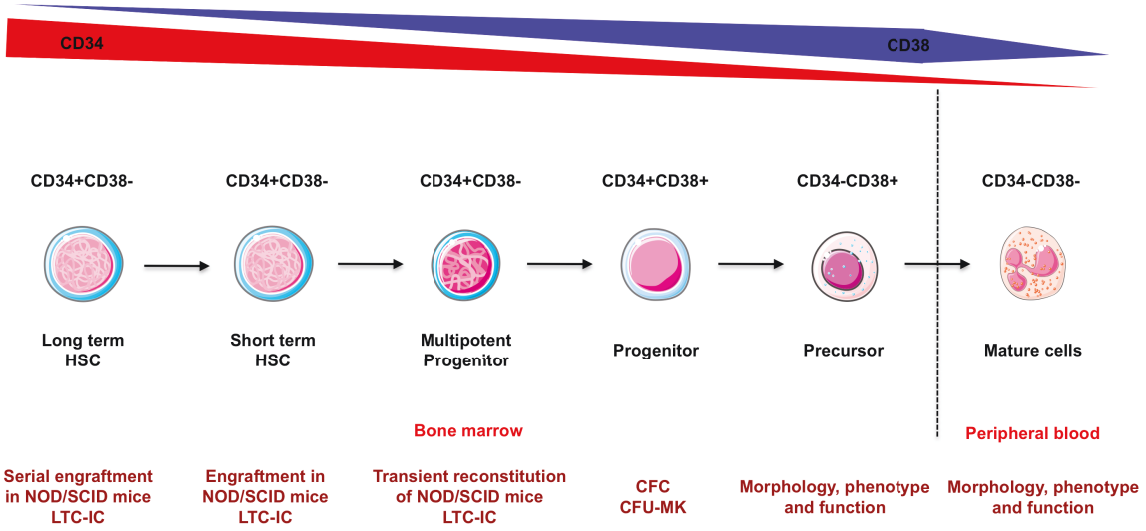

Figure 3 : Hematopoietic compartments. Diagram showing the different stages of hematopoietic stem cell differentiation, their location, the functional assays used to identify these cells, and the associated expression of CD34 and CD38.

In addition to morphological analysis, identification of the different hematopoietic compartments may be supplemented by surface antigens analysis. Indeed, all the cells in the body carry on their surface specific proteins, that can be used as markers to detect cell lineage and maturity. These surface antigenic markers are readily detectable by flow cytometry with the use of specific monoclonal antibodies coupled to fluorochromes. At present, there is still no specific and exclusive marker for HSCs. However, some markers are expressed during early stages of hematopoiesis and have been used in this regard. This is the case of CD34, which is very frequently used for human HSCs identification. However, its use is highly controversial because its expression has also been observed on the surface of endothelial cells[17] and stromal cells precursors.[18] Consequently, it is often combined with other markers of positive or negative selection such as CD38.[19] It is generally accepted that the most immature human hematopoietic cells express CD34 but not CD38. During differentiation, the human hematopoietic cells progressively lose the expression of CD34,[20]

and gradually acquire the expression of CD38 (until precursor compartment) as well as other lineage specific markers. If we only refer to the expression of CD34 and CD38 markers, it is generally accepted that HSCs express CD34 but not CD38 ($CD34^+$, $CD38^-$), the progenitors express CD34 and CD38 ($CD34^+$, $CD38^+$), the precursors have lost the expression of CD34 but keep that of CD38 ($CD34^-$, $CD38^+$) and finally, the mature cells have lost the expression of these two markers ($CD34^-$, $CD38^-$) (Figure 3). In the early 1990s, a combination of surface antigenic markers was first proposed to refine identification and isolation of HSCs.[21] In mice, this population expresses Sca-1 (stem cell antigen 1) but not the differentiation markers associated with B, T, and myeloid lineages, grouped under the abbreviation Lin (Lineage). In addition, these cells express low levels of Thy-1 (Thymocyte Antigen 1). This population was called Thy-1^{low}, Sca-1^+, Lin^-.[21] In humans, the closest cell population to HSCs was described as Thy-1^+, $CD34^+$, Lin^-.[22] LT-HSC are currently described as the Lin^-, $Sca1^+$, c-Kit^+, $CD45^+$, Thy1.1^{low}, $Flk2^-$, $CD34^+$, $Slamf1^+$ cell population in the murine hematopoietic system, and as the Lin^-, $CD34^+$, $CD38^-$, $CD45RA^-$, $CD90^+$, $CD123^+$, $CD133^+$ cells population in the human hematopoietic system.[21,23–26] In humans, the Lin^-, $CD34^+$, $CD38^-$, $CD90^+$, $CD123^+$, $CD133^+$ fraction is enriched in HSCs, but probably still contains one or more mature sub-populations. However, this remains technically difficult to demonstrate. This area of research is very dynamic, and research groups regularly propose new antigenic surface markers to refine the characterization of HSC population. For example, CD49f was recently proposed, as the loss of its expression is associated with a decrease in hematopoietic repopulating capability of a $CD34^+$, $CD38^-$, $CD45RA^-$, $Thy^{+/-}$ cell population.[27]

HSC fate is tightly controlled through many signals provided by their bone marrow microenvironment, the so-called stem cell niche. The niche allows contacts between stem cells and all the necessary factors to maintain their immaturity, such as extracellular matrix or cytokines.

IV. Hematopoietic niche

1. Concept

The niche is the environment in which stem cells reside. It is responsible for the maintenance of unique stem cell properties such as self-renewal, quiescence and stemness. The concept of "niche" emerged in 1978 from a work that demonstrated the existence of spatially defined zones capable of providing all the necessary factors for the survival and development of cells involved in tissue maintenance and regeneration in adult organisms.[28] This was further supported by the crucial role of bone marrow stromal cells for *in-vitro* cultivation of LTC-IC.[9] In bone marrow, HSCs are located in hematopoietic niches that are

complex three-dimensional structures containing extracellular matrix (ECM) proteins, soluble factors such as cytokines or growth factors, as well as stromal, endothelial and hematopoietic cells responsible of their synthesis.

2. Composition

The bone marrow stroma usually refers to tissue not directly involved in the marrow's primary function of hematopoiesis. This stroma is mainly composed of fibroblasts, myofibroblast, adipocytes, osteoblasts, chondrocytes, endothelial cells, osteoclasts and macrophages dispersed in ECM.[29] However, these different cell types do not have the same origin. Indeed, fibroblasts, myofibroblasts, adipocytes, chondrocytes, osteoblasts and reticular cells are derived from the differentiation of mesenchymal stem cells (MSCs) while endothelial cells originate from endothelial stem cells and macrophages are part of the hematopoietic lineage. Interestingly, nestin^{+} MSCs are spatially associated with HSCs and adrenergic nerve fibers in the bone marrow.[30] It has been demonstrated that adrenergic signals are able to induce MSCs differentiation toward osteoblastic lineage, thus leading to an increase in HSC content. Osteoclasts result from the fusion of macrophages in the presence of RANKL (receptor activator of nuclear factor κβ ligand) and M-CSF (Macrophage colony-stimulating factor).[31] Stromal cells have the potential to control hematopoiesis via soluble molecules or ECM synthesis, and through direct interactions with HSCs. Moreover, these factors are also produced by hematopoietic cells themselves, which are part of the bone marrow microenvironment.

3. HSCs regulation

HSC fate has been demonstrated to be dependant on many different factors, such as cytokines, ECM composition, interactions with other cells, glucose and oxygen levels or physical factors (Figure 4). Thus, hematopoiesis control is a very complex system because of the interplay between the different parameters.

To date, numerous cytokines and growth factors are known to regulate hematopoiesis.[32] Due to the large number of these factors and their multiple implications in hematopoiesis, it is difficult to make an exhaustive list. That is why only the main factors identified to date will be developed here. IL-6 (interleukin 6), SCF (stem cell factor), Flt3-ligand (Fms-like tyrosine kinase receptor-3) and LIF (Leukemia Inhibitory Factor) have been described as the principle factors affecting the most immature hematopoietic cells fate. They are able to increase the number of HSCs in cell cycle and to sensitize them to the action of other growth factors. IL-3 and GM-CSF (Granulocyte Macrophage-Colony Stimulating Factor) have been involved in survival and differentiation of hematopoietic progenitor cells.

Among these cytokines, GM-CSF is produced by several cell types such as T lymphocytes, monocytes, endothelial cells, fibroblasts or osteoblasts. GM-CSF primarily acts on proliferation and differentiation of granulocytic and macrophage lineages, but is also capable to induce differentiation of megakaryocytes, eosinophils, or erythrocytes in presence of erythropoietin. G-CSF (Granulocyte-Colony Stimulating Factor), M-CSF (Macrophage-Colony Stimulating Factor), IL-4, IL-5, IL-6, EPO (Erythropoietin) and TPO (Thrombopoietin) target more committed cells and promote proliferation and terminal maturation of these cells. Although TPO was initially considered as a factor influencing the cells engaged towards megakaryocytic differentiation, it was recently demonstrated that it could also induce HSCs proliferation. EPO is mainly produced by interstitial cells of the kidney and is specifically involved in erythroid precursors stimulation and globin synthesis. Finally, stromal cells also produce factors able to inhibit hematopoiesis, by regulating self-renewal, proliferation and differentiation of HSCs. Among them, there are particular types of prostaglandins E and some cytokines such as IL-8, MIP-1α (Macrophage Inflammatory Protein-1α), TNF-α (tumor necrosis factor α) and IFNs (Interferons).[33] Several key signaling pathways including Hedgehog, Notch, Wnt, TGF-β (Tumor Growth Factor β) and others play key roles in HSC biology. The TGF-β superfamily is composed of a multitude of molecules that include Bone Morphogenetic Proteins (BMPs), family particularly involved in HSC regulation and whose effects on these cells will be described in chapter 4.

ECM contains fibers of collagen, fibronectin, laminin, or proteoglycans that contribute to the hematopoietic niche structure and regulation. Hematopoietic stem cells have direct and relatively specific interactions with ECM and stromal cells in the bone marrow. These interactions are primarily responsible for adhesion and thus the maintenance of HSCs in the bone marrow. However, they also allow the transmission of signals that modulate the survival, proliferation and cell differentiation. Primary hematopoietic cells express over 20 different adhesion receptors.[34,35] Among these molecules, the best known belong to the integrin family. Integrins are transmembrane glycoproteins consisting of two subunits α and β, which, after binding with their ligand, stimulate different intracellular signaling pathways. Integrins are responsible for cell adhesion to ECM proteins such as fibronectin, laminin or collagen, or to stromal cells through cell adhesion molecules (CAM) such as ICAM and VCAM,[36] selectins, molecules of the "immunoglobulin-like" family such as that PECAM-1 or sialomucins such as CD34, CD43, CD45RA, PSGL 1 (P-selectin glycoprotein ligand-1) and CD164. HSCs mainly express α4β1 (VLA-4) α5β1 (VLA-5) integrins, the expression of which decreases with HSCs differentiation and is necessary for their progressive detachment from the bone marrow stroma.[37] Many studies have described the importance of these two integrins in regulating HSCs fate. Indeed, α4β1 integrin has been involved in the control of myelopoiesis, erythropoiesis and lymphopoiesis B.[38] This same integrin also controls HSCs

quiescence by maintaining its adhesion to the bone marrow stroma.[39]

While many different biological parameters governing HSC control have already been identified, some of them seem more complex and may not be as well understood. For example, in zebra-fish embryo, it was demonstrated that HSCs production depends on glucose levels in a dose-dependant response.[40] Moreover, a very interesting study also demonstrated that sympathetic nervous system regulates the attraction of HSCs to their niche.[41] This was revealed by the use of mice deficient in UDP-galactose ceramide galactosyltransferase, an enzyme required for normal peripheral nerve myelination and conduction, that failed to mobilize hematopoietic progenitors in response to G-CSF.

Other than biological factors, chemical composition or physical parameters have also been identified for their role in SC and HSC fate control. For example, hypoxia (low oxygen levels) has been shown to favor the self-renewal of both murine and human HSCs.[42–44] The primordial role of hypoxia in HSC control was further supported by demonstration that HSC are predominantly located in hypoxic regions of the bone marrow. This was evidenced by *in-vivo* perfusion of a Hoechst 33342 dye gradient simulating the level of oxygenation in the bone marrow.[45]

Biomechanical forces are critical regulators of embryogenesis. However, little is known about the role of mechanical forces in the regulation of adult SC. Interestingly, recent studies have demonstrated that these signals could be involved in HSC regulation.[46] Indeed, the application of shear stress on mouse embryonic stem cells led to the hematopoietic commitment of these cells. It was further evidenced that fluid shear stress increases the expression of *Runx1* in hematopoietic progenitor cells, leading to an increase of their colony-forming potential *in-vitro*.[46] The consequences of mechanical stress on HSC fate will be further described in chapter 3.

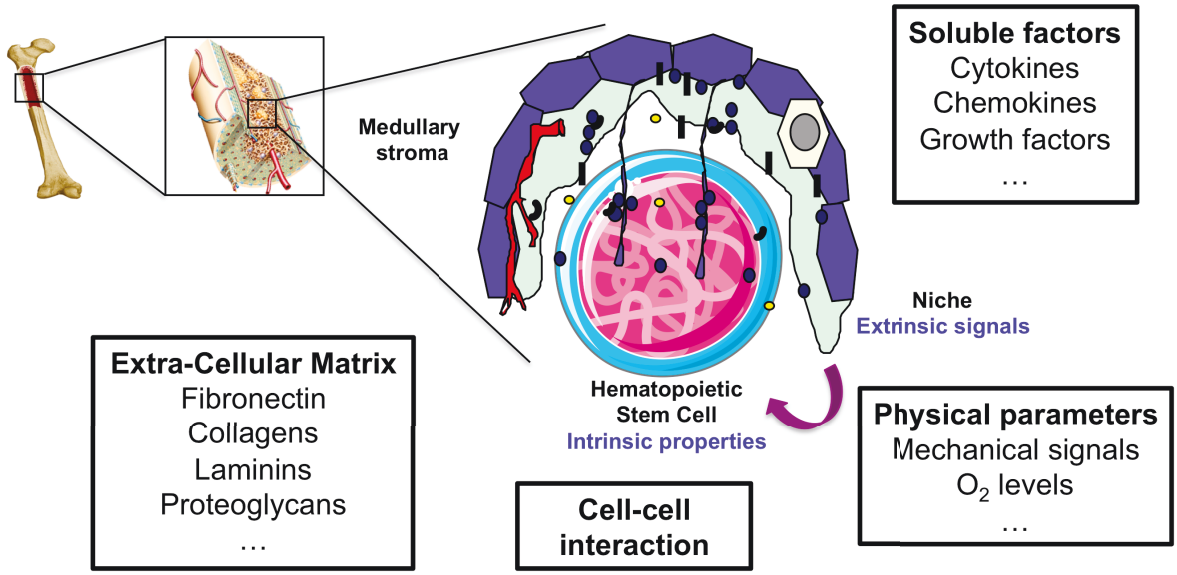

Figure 4 : Hematopoietic stem cell fate control by their microenvironment. HSC fate is tightly controlled through interactions with their bone marrow microenvironment, the so-called hematopoietic niche. This dialogue is mediated by different factors such as soluble factors, extra-cellular matrix, cell-cell interactions as well as physical parameters. In a physiological context, all those parameters allow a fine control of hematopoiesis.

Interestingly, the different components of the microenvironment seem to respect a precise distribution within the bone marrow, suggesting that their interactions with hematopoietic cells may dictate the location of the latter.[47] Indeed, it has been demonstrated that interactions between HSCs and their microenvironment depend on the hematopoietic cell lineage and its degree of maturation.[48] According to the current hypothesis, the specialized region within BM that houses and controls HSCs fate has been classified into two morphologically different niches: osteoblastic niche (or endosteal niche) and vascular niche (or endothelial niche). HSCs are mainly maintained by the osteoblastic niche, which provides a quiescent HSC microenvironment, while the vascular niche is thought to regulate HSCs proliferation, differentiation, and mobilization.

4. Osteoblastic niche

Osteoblastic niche is precisely located at the interface between the bone and the bone marrow, where new trabecular bone is formed by osteoblasts (Figure 5).[49] By definition, this niche mainly consists in osteoblasts that are involved in the control of HSC fate (Figure 6). Unlike osteoclasts that resorb bone matrix, osteoblasts synthesize it, thus ensuring a constant remodeling of bone tissue. The tightly regulated balance between these two cell populations maintains bone tissue homeostasis. In 2003, Zhang's group developed a mouse model invalidated for the BMP type Ia receptor (BMPRIa), a receptor usually expressed at the membrane of osteoblasts.[50] Using this model, they demonstrated that BMPRIa loss

increased the number of osteoblasts in the mice bone marrow, and that it was correlated with an increased number of HSCs. Further analysis allowed them to demonstrate a real interaction between osteoblasts and HSCs through adherent junctions involving N-cadherin. The same year, these observations were supported by the development of a transgenic mouse model producing osteoblast-specific, activated PTH/PTHrP receptors, constitutively active for parathyroid hormone signaling.[51] This induced the production of high levels of Notch and Jagged1 ligands and the proliferation of osteoblasts, thus leading to an increase in the number of HSCs. In 2004, the importance of Tie-2/Angiopoietin1 interaction in HSCs regulation was also demonstrated. Tie-2 is expressed by quiescent murine HSCs, localized near the bone surface, at the location described by Zhang's group as the osteoblastic niche.[50] By using an *in-vitro* co-culture system with OP9 murine stromal cells, the authors demonstrated that Tie-2/Angiopoietin1 interaction increases HSCs adhesion to the stroma and maintains their immature phenotype by inhibition of cell division and induction of quiescence.[52] This leads to the maintenance of HSCs' repopulation capabilities *in-vivo*. Osteoblasts are involved in the production of numerous ECM proteins such as type I collagen fibers and fibronectin. Nevertheless, in 2005, two studies based on the use of a mouse model deleted for osteopontin, an ECM protein highly expressed in bone, have demonstrated the importance of this glycoprotein in the regulation of HSC, with whom it interacts via CD44, VLA-4 and VLA-5 (Very Late Antigen-4/5) molecules.[53] It was demonstrated that an osteopontin-free environment is able by itself to increase the number of HSCs and to reduce their apoptosis, suggesting that osteopontin is able to control the size of HSCs population *in-vivo*. A second study reported that osteopontin is mainly localized close to the bone surface and that it plays an important role in the localization of murine HSCs after transplantation.[54] These studies also demonstrated that osteopontin physically interacts with HSCs in this particular area, thus regulating their proliferation. *In-vitro*, osteopontin decreases the proliferation and differentiation of murine and human hematopoietic progenitors but does not induce apoptosis of $CD34^+$, $CD38^-$ cord blood cells.[54]

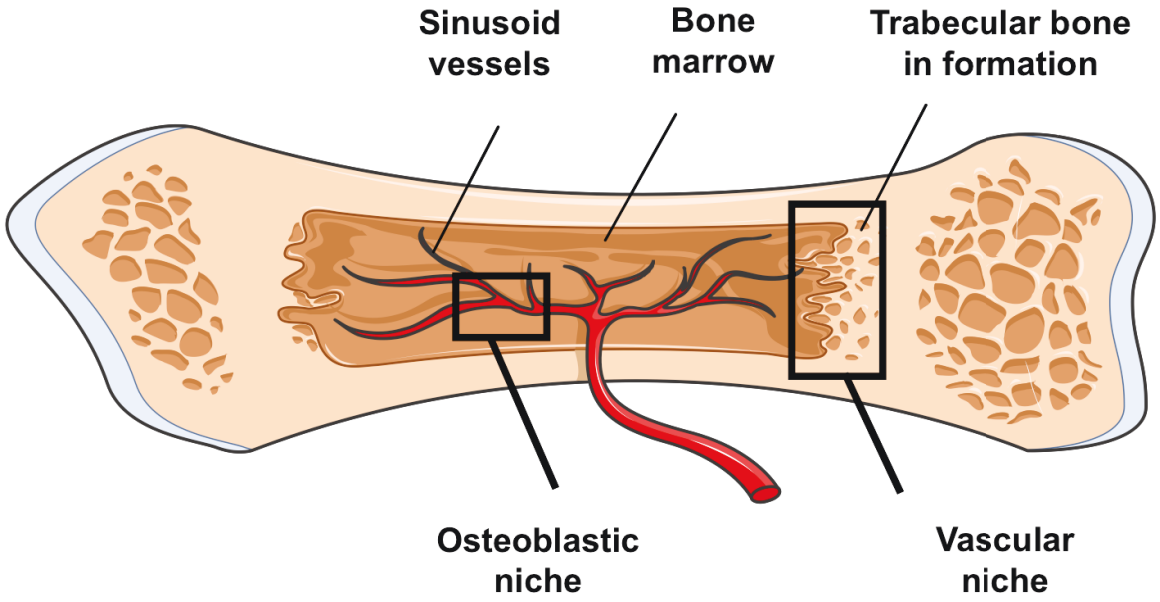

Figure 5 : Location of osteoblastic and vascular niches. Hematopoietic microenvironment is composed of two structurally distinct niches. Osteoblastic niche is precisely located at the interface between the bone and the bone marrow, where new trabecular bone is formed by osteoblasts. Vascular niche is located in the axial part of the bone at the center of the bone marrow, a heavily vascularized surface with endothelial cells lining the sinusoid vessels and reticular cells.

5. Vascular niche

The existence of vascular HSC niche has been demonstrated in 2005.[55] It is located in the axial part of the bone at the center of the bone marrow (Figure 5). Since the endosteal surface is heavily vascularized, vascular and perivascular cells, such as reticular cells, adipocytes, and mesenchymal cells might contribute to the formation of HSC niches at or near the endosteum. In the vascular niche, HSCs are located near reticular cells and endothelial cells lining the sinusoid vessels, allowing exchanges with the venous circulation (Figure 6).[55] However, HSCs regulation by the vascular niche has been less documented compared to their regulation by osteoblastic niche. Nevertheless, it has been reported that sinusoids near which are located HSCs are surrounded by reticular cells that secrete abnormally high levels of SDF1 (Stromal cell-Derived Factor-1), a molecule involved in the maintenance and mobilization of HSCs.[56] More recently, co-culture of mouse HSCs with bone marrow endothelial cells showed that by direct cell contacts, endothelial cells were able to maintain LT-HSCs in culture and to stimulate their expansion while retaining their self-renewal capabilities.[57] *In-vivo* studies using a murine model deleted for Notch1/Notch also showed that bone marrow endothelial cells are responsible for the proliferation of LT-HSCs through Notch signaling.[57] Furthermore, in adult mice, conditional deletion of VEGF receptor type R2 (VEGFR2), which prevents the regeneration of reticular cells surrounding the

vascular niche, also prevents hematopoietic reconstitution of irradiated mice.[58] Moreover, the selective activation of Akt1 (c-akt murine thymoma viral oncogene homolog 1) in bone marrow endothelial cells led to an increase in the number of HSCs and to an acceleration of the hematopoietic repopulation after myeloablation (severe or complete depletion of bone marrow cells) in adult mice.[59] These observations demonstrate that bone marrow endothelial cells as well as neighboring reticular cells play an important role in the regulation of HSC, particularly for HSCs maintenance and localization.

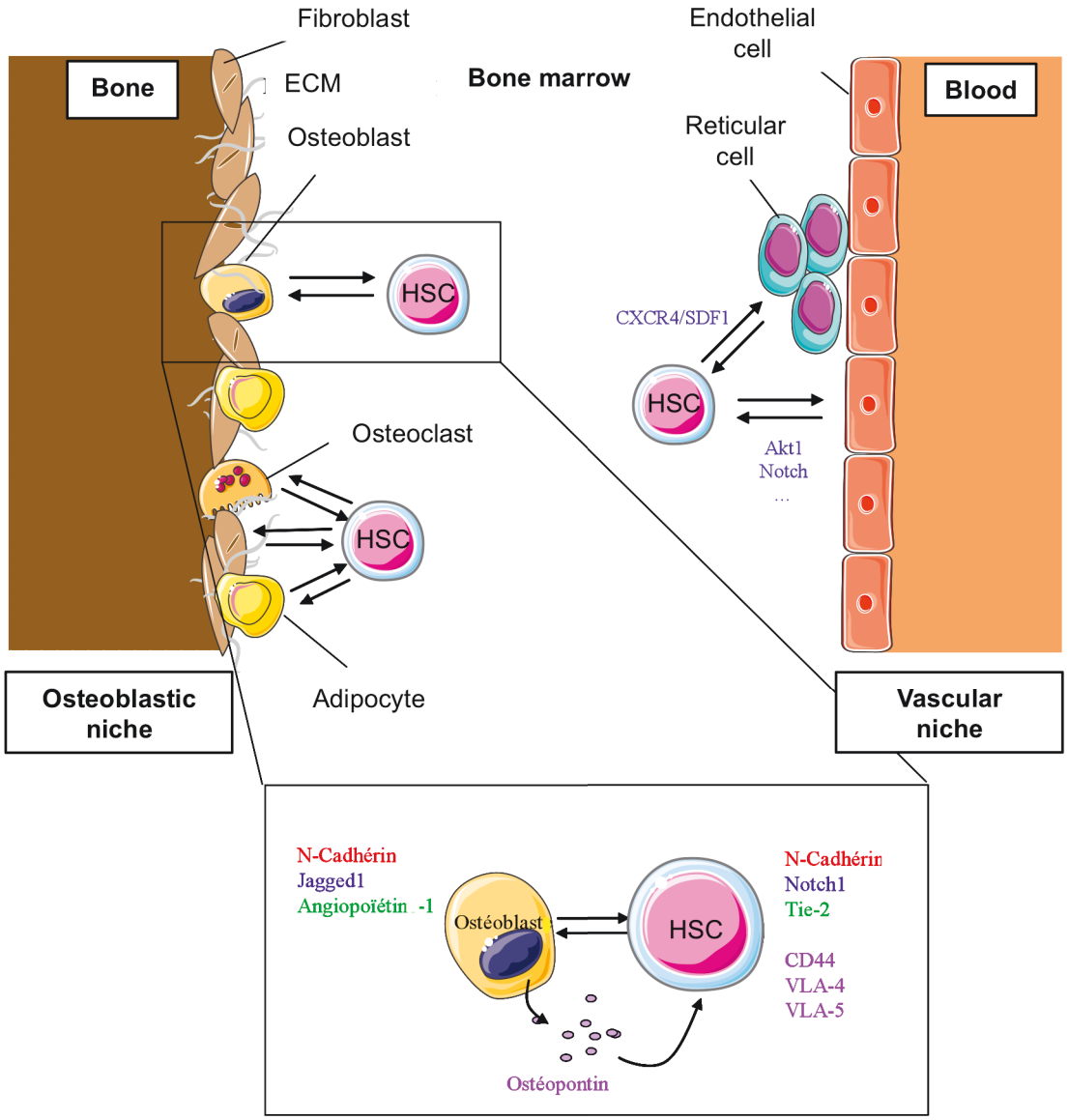

Figure 6 : Regulation of HSCs by osteoblastic and vascular niches. In the vascular niche, HSCs interact with endothelial cells that form the blood vessels via the Notch signaling pathway or proteins such as Akt1. HSCs also interact with reticular cells via SDF1/CXCR4 signaling. In the osteoblastic niche, a large number of different cell types coexist. However, osteoblasts have been described as the main regulators of HSCs. HSCs and osteoblasts interact through N-cadherin, Jagged/Notch1, Angiopoïétin-1/Tie-2 and osteopontin signaling.

The intricate process of hematopoiesis is tightly regulated by the BM microenvironment to ensure the proper generation of sufficient blood cells. Therefore, defects in self-renewal and differentiation can severely hamper normal hematopoiesis, leading to hematopoietic insufficiency or leukemic processes. In the case of leukemia, these aberrations are often the result of inherited or acquired genetic alterations.

V. Leukemia

Leukemia is a hematologic disorder characterized by the abnormal increase in the number of white blood cells in the tissues. It is responsible for an estimated 4% of all cancer-related deaths. This cancer of the blood tissue is classified according to the phenotype of dominant proliferating cells and to the speed of disease progression. Acute leukemias are characterized by the rapid increase of large numbers of immature blood cells. This huge production of immature blood cells impairs the production of normal cells. Due to their immaturity, these cells are unable to function properly to prevent or fight infection. Inadequate numbers of red blood cells and platelets being made by the bone marrow cause anemia, as well as easy bleeding and bruising. Furthermore, due to overcrowding of the bone marrow, these immature blood cells, called blasts, can enter the circulation and spread throughout the body. Chronic leukemias are characterized by a slower accumulation of more mature blood cells as compared to acute leukemias. Since acute and chronic leukemias can affect cells belonging to the myeloid or lymphoid lineages, leukemias are divided into four main categories: Chronic Myeloid Leukemia (CML), Acute Myeloid Leukemia (AML), Acute Lymphoblastic Leukemia (ALL) and Chronic Lymphocytic Leukemia (CLL). Leukemia can be further classified based on the cell type involved such as B cell Chronic Lymphocytic Leukemia (B-CLL) or T cell Acute Lymphoblastic Leukemia (T-ALL). In addition, lymphomas are also cancers derived from the lymphocytic lineage. However, the main difference between leukemias and lymphomas is that in leukemia, cancer cells are mainly present in the bone marrow and blood, while in lymphoma it tends to be in lymph nodes and other tissues. As the focus of this project, CML biology will be detailed in Chapter 2.

ALL is the most common form of cancers in children and represents approximately 25% of cancer diagnoses among children younger than 15 years. Nevertheless, it can also appear in adults. It is characterized by an over-production of cancerous immature cells called lymphoblasts. Based on the expression of lineage-specific antigens and the presence of lineage-specific gene rearrangements, ALL cells has been identified to be derived from either B- or T-cell precursors.[60] The remission rate for ALL has improved dramatically over the past few decades, due to the development of chemotherapies and the use of stem cell

transplantation. Indeed, the 5-year event-free survival has improved from 10% in the 1960s to about 90% these days.[61] Up to now, different factors have been associated with an increased risk of ALL, such as high doses of radiations, or genetic syndroms such as Down syndrom or Ataxia telangiectasia.

CLL is a monoclonal disorder characterized by a progressive accumulation of non-functional lymphocytes. CLL is the most common form of leukemia in adults and constitutes 28% of all leukemia cases, with an incidence of 1 to 5 per 10^5 people worldwide.[62] CLL affects mostly elderly patients. Unlike ALL that is characterized by a sudden burst of leukemic cells, CLL leukemic cells tend to build up over time, and many people do not have any symptoms when it is diagnosed. Wide range of genetic variations like hypermutation of the immunoglobulin heavy-chain genes (IGHV) or genomic aberrations reflect the clinical and biological heterogeneity of the disease.[63]

AML occurs more frequently in adults than in children. This is highlighted by a median age at diagnosis of 63 years. While AML is characterized by the rapid expansion of abnormal blasts of the myeloid lineage, it is a very heterogeneous disease. Indeed, AML classification is defined on the basis of the presence of specific chromosomal and/or molecular alterations. The 2 systems commonly used in the classification of AML are the French-American-British (FAB) system and the World Health Organization (WHO) system. The FAB system discriminates AML into 8 different sub-types based on morphology and cytochemistry.[64] The WHO classification was introduced to include newer prognostic factors such as molecular markers and chromosome translocations. The WHO system identifies 4 AML subgroups: AML with recurrent genetic abnormalities, AML with multilineage dysplasia, therapy-related AML and myelodysplasic syndromes, and those that do not fall into any of these groups. These 2 systems created a minimum of 17 subclasses of AML, allowing physicians to identify subgroups of patients with different prognosis and who might benefit from specific treatment strategies. Thus, the 5-year survival can range from 3% to more than 80% depending on the AML sub-type.[65] Several factors have been associated with an increased risk of AML, such as high doses of radiations, benzene exposure, treatment with some chemotherapeutics drugs or Down syndrom.

The existence of a small population of leukemic cells that are alone capable to initiate leukemia and to reconstruct all the different existing subpopulations within the tumor was first demonstrated in AML.

VI. Leukemic Stem Cells

Leukemic Stem Cells (LSCs) were first identified by the observation that a $CD34^+$, $CD38^-$ AML leukemic cell subpopulation alone is capable of engrafting after injection into NOD/SCID immunodeficient mice, thus producing a disease similar to human AML.[66] This small fraction of leukemic cells alone was able to proliferate extensively both *in-vitro* and *in-vivo*. Indeed, the extended self-renewal capability of these cells was demonstrated by successful engraftment into secondary recipient mice. First referred as leukemia initiating cells, these cells were later described as LSCs or cancer stem cell (CSCs). This minor fraction in the tumor retained (or acquired) the self-renewal and proliferation characteristics of normal stem cells and is alone capable to reconstruct all the different existing subpopulations within the tumor.[67] These observations suggest that leukemic transformations occur in primitive normal hematopoietic cells, which then give rise to leukemia through clonal expansion (Figure 7). On the other hand, the leukemic transformation may also occur by re-acquisition of self-renewal capabilities by committed normal progenitors that will then be able to initiate leukemia (Figure 7).[67]

Although this concept is still widely discussed, numerous studies have since demonstrated the existence of such subpopulations in almost all types of cancer including hematopoietic, brain, bladder, liver, lung, skin, ovary, pancreas, prostate, colon and breast cancers.[66,68–77]

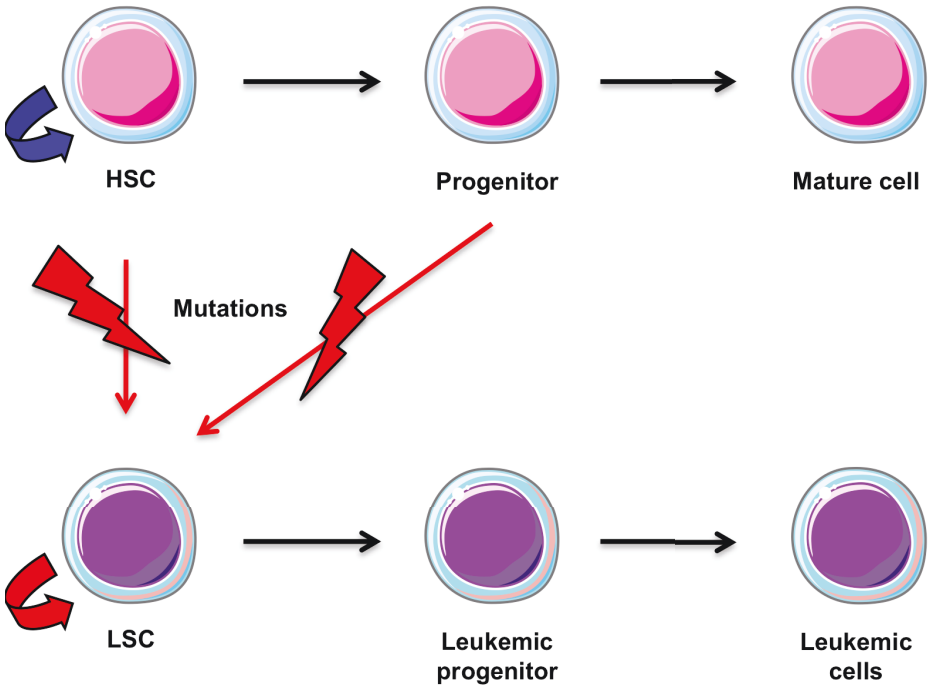

Figure 7 : Model of leukemic stem cell transformation. Leukemogenesis can occur by acquisition of genetic abnormalities in a HSC that give rise to a LSC with self-renewal capability. Alternatively, it can happen in a progenitor cell that will re-acquire self-renewal capability.

The concept of CSC is derived from the similarities that exist between normal SCs and CSCs. The signaling pathways used by both normal SCs and CSCs overlap and are based on embryonic signaling pathways allowing self-renewal capabilities. However, while normal SCs fate is tightly controlled, CSCs display increased proliferation capabilities, thus leading to tumor growth. Although the concept remains controversial, new observations from clinical studies and basic research have led to a more comprehensive CSC model of tumorigenesis, tumor recurrence, and metastasis formation. Located at the top of the tumor hierarchy, CSCs constitute a subpopulation of malignant cells capable of self-renewal and differentiation. Thus, they are able to generate all cell types present in a tumor, including non-CSC progeny, which form the tumor bulk. CSCs are described by their unique ability to engraft into immunodeficient mice, to recapitulate the tumor of origin both morphologically and phenotypically in xenografts, and to be serially transplanted. Just like normal HSCs, LSCs are usually rare and represent only a small cell fraction in the tumors. It has been demonstrated that about 1 in 10^2 to 10^4 AML leukemic cells are able to form colonies in CFC assay *in-vitro*, and only 1 to 4% of leukemic cells formed colonies in spleen after murine transplantation and were described as LSCs.[78] However, LSC-enriched populations have been reported to represent extremely variable proportions of bulk tumor cells, ranging from 0.2% to 82.5% in leukemia and lymphoma genetic mouse models.[79] Moreover, the frequency of CSCs might increase during tumor progression, as recently shown by *in-vivo* limiting dilution assays comparing grade 1 and grade 3 breast tumors.[80] LSCs, which are considered to originate from hematopoietic stem or progenitor cells, not only adopt the regulatory machinery operating in normal HSCs but establish their own mechanisms against apoptosis and senescence.

Clinically, the CSC discovery predicts that if the eradication of tumor cells induces remission, only the destruction of CSC can lead to a cure (Figure 8).[81]

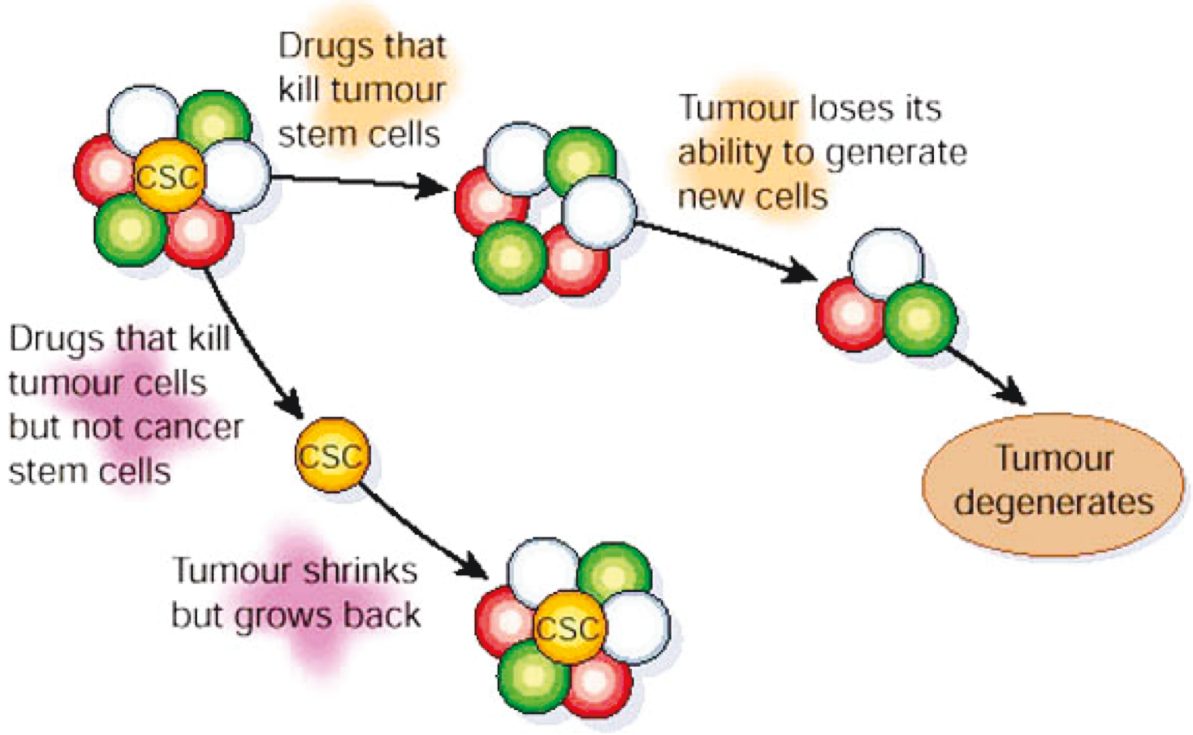

Figure 8 : Involvement of cancer stem cells in the re-emergence of the disease after treatment. While conventional therapies used in the treatment of cancers manage to kill most of the cancer cells, they are ineffective against CSCs. After treatment discontinuation or upon accumulation of genetic abnormalities, these cells will gradually allow the reconstitution of the tumor mass and the gradual resurgence of the disease. This predicts that only the destruction of CSCs can lead to a cure in cancers.

VII. Cancer Stem Cells and resistance to treatment

Most of the treatments used in cancer are based on cytotoxic molecules targeting rapidly cycling cells, one of the main properties of cancer cells. However, these treatments are problematic for 2 different reasons. The first one is that chemotherapy also harms normal cells that rapidly divide under normal circumstances like bone marrow, digestive tract and hair follicles cells. The second one is that CSCs, or at least some of them, are able to survive to this treatment. These resistant CSCs can persist during treatment and will eventually acquire additional alterations leading to relapses of the disease in patients (Figure 8). Different mechanisms have been identified for their involvement in CSCs resistance to treatment. One of them is the possibility for CSCs to enter into quiescence. In that case, the cells are not dividing and will not be sensitive to chemotherapeutic treatments. Other mechanisms are dependant on specific SC properties that allow these cells to survive all along an individual's lifetime, such as high DNA repair ability, over-expression of anti-apoptotic proteins, drug efflux transporters and detoxifying enzymes.[11]

Finally, CSCs are also supported by their microenvironment for their survival and can even subvert the tumor niche to resist to treatments.

VIII.Cancer stem cell niche

Just like normal SCs, CSCs are located in a peculiar microenvironment. However, the response of CSCs to the signals provided by their microenvironment can be totally altered. Indeed, in a healthy context, these signals are sufficient to control SC fate, while in cancer they are not able to control CSCs fate as cancer is characterized by an abnormal amplification of malignant cells. For example, it has been demonstrated that CML $CD34^+$ cells respond to hypoxia with a reduction in division number while this is not the case for normal $CD34^+$ cells.[44] Moreover, several studies have demonstrated that tumor cells can alter their microenvironment, leading to the production of abnormal signals that can even contribute to the tumor phenotype.[82] This has been particularly well illustrated in ALL, where leukemic cells dissemination into the BM of irradiated mice lead to the alteration of the normal microenvironment, depending on the level of leukemic cell engraftment.[82] It was further demonstrated that in response to chemotherapy, leukemic cells were able to recruit mesenchymal cells by production of a set of cytokines, including GDF-15, a member of the TGF-β signaling pathway. This led to the formation of a protective microenvironment and to the resistance of ALL leukemic cells under chemotherapy.[82] In addition, the alterations of the microenvironment by leukemic cells can even lead to the disruption of normal hematopoietic cells behavior, as demonstrated by the analysis of BM microenvironment after transplantation of healthy human $CD34^+$ cells in a ALL mouse xenograft model.[83] Current evidences demonstrate that homeostatic processes such as inflammation, hypoxia and angiogenesis contribute to the maintenance and control of CSCs fate by providing the appropriate signals within the microenvironment.[84] As the niche is involved in the protection and the maintenance of normal SCs, an increasing amount of evidences also indicates that it could also be involved in CSCs resistance to anti-cancer treatments.[82,85]

Cancer remains one of the leading causes of mortality worldwide. One of the main causes of treatment failure in cancers is the development of drug resistance by cancer cells. The persistence of cancer stem cells (CSCs) might explain cancer relapses as they could allow reactivation of cancer cells proliferation following therapy, leading to disease persistence and ultimately to patients' death. Clinically, it is crucial to develop therapeutic strategies able to target resistant CSC in order to cure the patients (Figure 8). Thus, it is necessary to understand the key processes occurring within the CSC niche to identify potential therapeutic targets that can serve as the basis for development of more effective treatments. In this context, Chronic Myelogenous Leukemia (CML) is the reference model of a true HSC alteration by a t(9;22) chromosomal translocation directly responsible of the leukemic transformation.[86] Thus, CML represents a unique model to study LSC biology and to understand the mechanisms of CSC transformation and resistance to treatments.

Chapter 2 : Chronic Myelogenous Leukemia

I. Introduction to CML

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder resulting from the clonal expansion of a transformed multipotent HSC.[87] CML is characterized by the t(9;22)(q34;q11) reciprocal chromosomal translocation, which leads to the formation of the *Bcr-Abl* oncogene, directly responsible of the leukemic transformation (Figure 9).[88] BCR-ABL fusion protein is a hyperactive and deregulated tyrosine kinase that promotes leukemic growth by disrupting a broad range of signaling pathways involved in cell survival, proliferation, and differentiation.[86] The molecular basis for the myeloid expansion is puzzling as BCR-ABL transforms HSC and is present in all hematopoietic compartments including myeloid, erythroid, B-lymphoid and T-lymphoid cells.[67] By studying female patients with X chromosome linked heterozygous alleles, CML was demonstrated to be a clonal disease that origins from a single multipotent HSC.[87,89] CML was the first cancer to be linked to a unique and clear genetic abnormality, the chromosomal translocation known as the Philadelphia (Ph) chromosome, so-called because it was first discovered and described in 1960 in Philadelphia.[90] This chromosomal abnormality is the hallmark of CML as it is found in about 90% of patients. The remaining 10% of CML patients have variant translocations that may be complex involving chromosomes 9 and/or 22 with one or more other chromosomes. Among them, 5% of CML cases display cytogenetic variants of the classic Ph chromosome. About 2.5% of the remaining CML patients are Ph chromosome negative but do have cryptic *Bcr-Abl* gene fusion detectable on molecular analysis. Hence, only about 2.5% of CML patients are negative for both the Ph chromosome and the *Bcr-Abl* fusion gene.[86] This unique genetic abnormality allowed the researchers to develop targeted therapies blocking BCR-ABL tyrosine kinase activity. Imatinib, the first tyrosine kinase inhibitor (TKI) developed, has proved to be very efficient to induce the remission of a majority of CML patients.[91] However, none of TKIs available to date seem to eradicate undifferentiated BCR-ABL$^+$ cells that may serve as a reservoir for additional oncogenic events leading to disease progression and requiring continued treatment.[92] Therefore, CML represents a unique model to study LSC biology and to elucidate some of the mechanisms of therapeutic resistance.

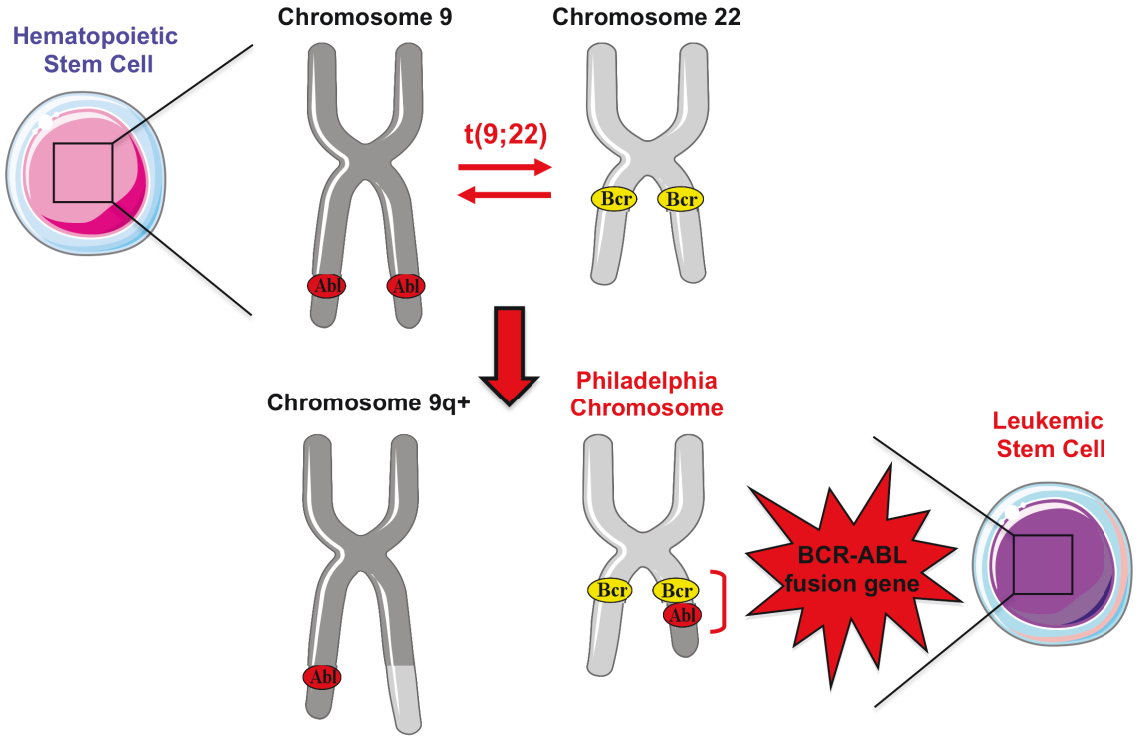

Figure 9 : BCR-ABL formation by chromosomal translocation in CML. The balanced genetic translocation t(9;22) characteristic of CML involves a fusion of the Abelson oncogene (*Abl*) from chromosome 9 with the breakpoint cluster region (*Bcr*) gene on chromosome 22 in a hematopoietic stem cell. This leads to the formation of the *Bcr-Abl* oncogene directly responsible of the leukemic transformation.

II. Epidemiology

CML constitutes about 15% of all leukemia and is considered a rare disease as its annual incidence rate vary from 0.6 to 2.0 cases per 100 000 inhabitants.[86] The incidence increases with age and is slightly higher in men than in women (men to women ratio of 1,4:1).[93] The prevalence is steadily increasing due to the substantial prolongation of survival that has been achieved by targeted therapies. CML occurs at any age, though very rarely in children as it represents only 5% of childhood leukemia. The median age at diagnosis is about 65 years.[94] However, the initiating event or events are still unknown: there are no known hereditary, familial, geographic, ethnic or economic associations. Benzene exposure has been identified as a cause of AML, but while some cases of CML have been reported in benzene-exposed workers, CML does not appear to be related to benzene exposure.[95] The only risk factor that has been associated with an increased CML occurrence is high levels of radiations. Indeed, follow-up of Hiroshima Japanese adult survivors after exposure to atomic bomb radiations revealed high rates of cancer, with an abnormally high peak in CML incidence 6 years after the accident. Correlation study between dose exposure and CML

occurrence suggests the existence of a threshold for CML development somewhere between 0.5 Gy and 0.09 Gy.[96]

III. BCR-ABL formation and structure

The balanced genetic translocation t(9;22)(q34;q11.2) characteristic of CML involves a fusion of the Abelson oncogene (*Abl*) from chromosome 9q34 with the breakpoint cluster region (*Bcr*) gene on chromosome 22q11.2. The molecular consequence of this translocation is the generation of a *Bcr-Abl* fusion oncogene, which in turn translates into a BCR-ABL oncoprotein.

1. Abl gene

The Abl proto-oncogene is the normal cellular homologue of the transforming gene of Abelson murine leukemia virus, a virus that causes pre-B cell leukemia in mice (Figure 10A). ABL is a 145 kDa protein that shows very low tyrosine kinase activity in the normal context. This protein is distributed in both the nucleus and cytoplasm of cells, and shuttles between the two compartments. It transduces signals from cell-surface receptors in response to growth factors and adhesion receptors to regulate cytoskeleton structure.[97] Abl can be activated by cell-cycle progression, DNA damage and integrin mediated adhesion. Abl tyrosine kinase is increased during cell-cycle progression to S phase. Activated Abl phosphorylates C terminal domain of RNA polymerase II and enhances transcription. In addition, Abl is activated in response to DNA damage and induces cell-cycle arrest or apoptosis.[98]

2. Bcr gene

The breakpoint cluster region (*Bcr*) gene leads to the formation of a 160 kDa protein, that can oligomerizes, autophosphorylates and transphosphorylates several protein substrates (Figure 10A).[99] BCR deficient mice develop normally but display excessive levels of oxygen metabolites produced by neutrophils following their activation.[100] Having different types of structural and functional properties, BCR protein may be involved in different signaling pathways and serve as the cross point of these pathways, but the understanding of the biological function of BCR protein is still limited.

3. Bcr-Abl isoforms

Depending on the precise location of the fusion, different sizes of the *Bcr* gene will be attached to the *Abl* gene, and distinct *Bcr-Abl* isoforms will be obtained (Figure 10A).

Consequently, the molecular weight of BCR-ABL can range from 185 to 230 kDa. This difference in structure influences the biological and clinical phenotypes associated with the *Bcr-Abl* variants. Three clinically important variants exist, referred as p185 (e1a2 junction), p210 (b2a2 or b3a2 junction), and p230 (e19a2 junction) isoforms, usually associated with distinct types of leukemia.[101] P185 is associated with 20% to 30% of ALL, p230 with a subset of patients with chronic neutrophilic leukemia (CNL) and p210 with 90% of CML. Still, the isoforms expression is not exclusive as p210 occurs in 40% of BCR-ABL$^+$ ALL, p185 occurs in 2% to 3% of CML, and p230 in a few cases of CML.[101,102] In addition, co-expression of p185 and p210 has been observed in some CML patients.[103] Different isoforms of p210 can also be produced by alternative splicing, namely, b2a2 and b3a2. However, there is no consensus on the biological consequences, as some studies suggested no difference in prognosis between patients with b2a2 versus b3a2 transcripts while other studies claimed the opposite.[104] In addition to the formation of the chimeric *Bcr-Abl* gene on chromosome 22, the reciprocal translocation also leads to the formation of the chimeric *Abl-Bcr* gene on chromosome 9.[105] Although transcriptionally active, very little is known about the biology of *Abl-Bcr* as it doesn't appear to have any functional role in CML. However, it has recently been suggested that *Abl*-Bcr could contribute to the leukemic phenotype in BCR-ABL$^+$ ALL by inducing B-cell commitment.[106,106]

4. p210 BCR-ABL structure

Structurally, BCR-ABL protein contains multiple domains (Figure 10B). The *Abl* sequences encode Src-homology (SH3 and SH2) domains, SH1 tyrosine kinase domain, DNA-binding domain, actin-binding domain, nuclear localization signals, and nuclear export signal. The *Bcr* region encodes a coiled-coil oligomerization domain, serine/threonine kinase domain, pleckstrin homology (PH) domain, a Dbl/cdc24 guanine nucleotide exchange factor homology domain, several serine/threonine and tyrosine phosphorylation sites, and binding sites for the Abl SH2 domain, Grb2, and 14-3-3 proteins.[107] The SH2 domain of BCR-ABL recruits signaling proteins such as p62dok, c-Cbl, and Rin1. Binding and phosphorylation of these molecules may be functionally important as SH2 mutations in BCR-ABL affect the course of disease in biological models. The actin-binding domain directly links BCR-ABL to the cytoskeleton, and facilitates tyrosine phosphorylation of cytoskeletal proteins.[108]

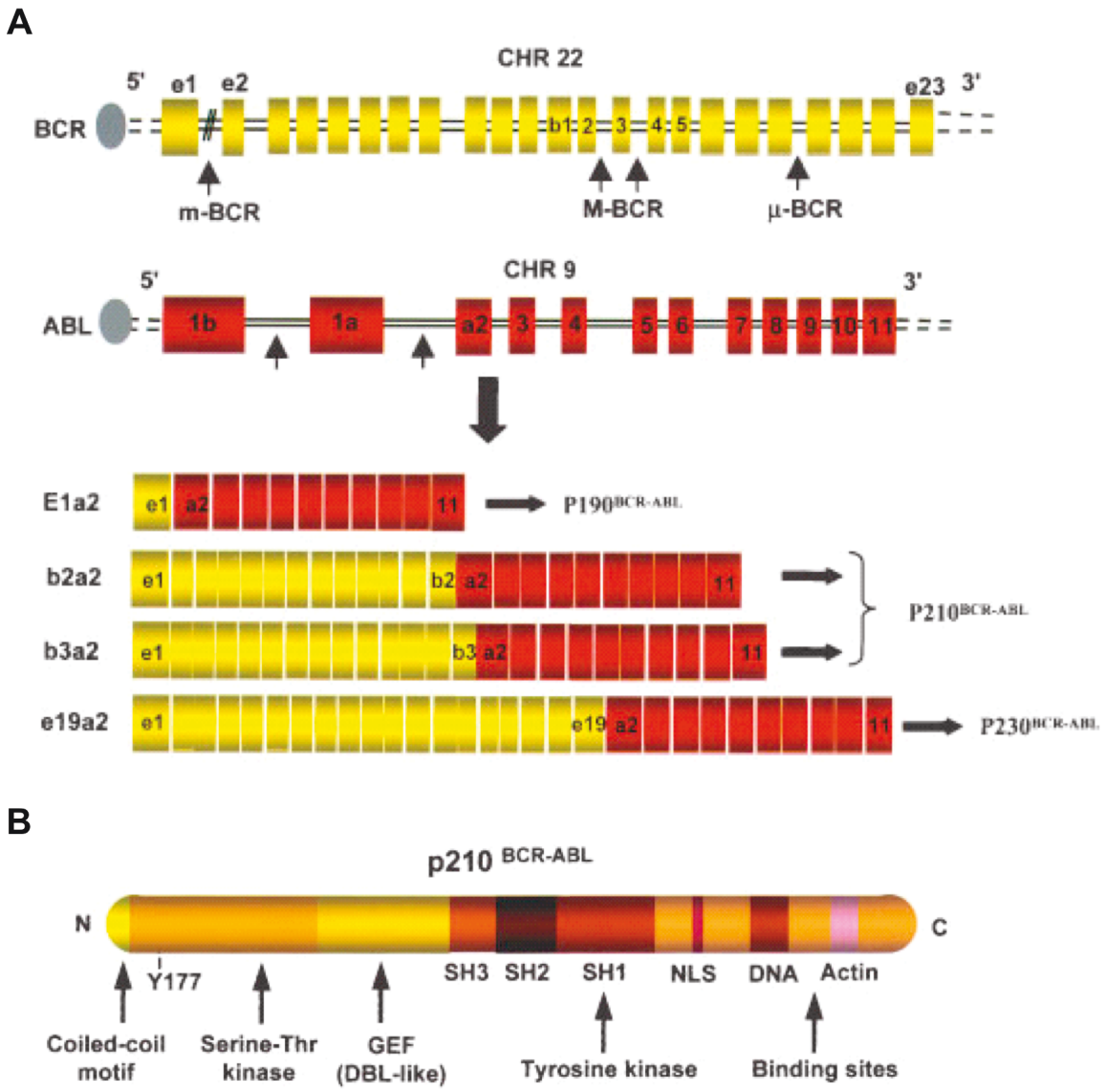

Figure 10 : Structural and functional domains of BCR-ABL. (A) Structure of *Bcr*, *Abl*, and *Bcr-Abl* isorforms. (B) p210 BCR-ABL contains a coiled-coil oligomerization domain at the N-terminus. Then there is a serine/threonine kinase domain, a Rho guanine nucleotide exchange factor (Rho-GEF) domain, a Src homology 3 (SH3) domain, a SH2 domain and a SH1 tyrosine kinase (TK) domain and a nuclear localisation signal (NLS). At the C-terminus are located DNA- and actin-binding domains.

IV. Molecular and functional consequences

1. Molecular consequences

BCR-ABL forms dimers or tetramers in vivo that autophosphorylate each other. Fusion of Bcr to Abl inhibits the latter's SH3 kinase regulatory domain, resulting in constitutive activation of the Abl tyrosine kinase in BCR-ABL. The BCR-ABL fusion protein acts as an onco-protein by recruiting adaptor molecules, phosphorylating signaling

molecules, and activating several signaling pathways that lead to cell transformation. Examination of the signaling pathways differentially regulated in CML led to a better understanding of BCR-ABL mediated leukemogenesis. BCR-ABL is involved in the alteration of diverse intracellular signaling pathways, including Ras-MAPK, PI3K-Akt, and JAK-STAT pathways.[109] BCR-ABL binds several adaptor proteins such as growth factor receptor bound protein 2 (Grb2), v-Crk avian sarcoma virus CT10 oncogene homologue-like (CrkL) protein and Src homology containing (Shc) protein. As BCR-ABL substrates represent a very extensive number of proteins, some examples will be discussed here to illustrate their functional consequences.

2. Functional consequences

Because of its effects on a wide range of cell signaling pathways, BCR-ABL expression profoundly alters HSCs functional properties. BCR-ABL is known to protect CML cells from apoptosis. Transduction of BCR-ABL in hematopoietic cell lines leads to an increase of anti-apoptotic genes expression, such as B-cell lymphoma-2 (*Bcl-2*), B-cell lymphoma-extra large (*Bcl-xL*) and myeloid cell leukaemia-1 (*Mcl-1*).[110] Thus, CML $CD34^+$ cells become more resistant to a large range of cytotoxic agents compared to their normal counterparts.[111]

Normal hematopoietic immature cells are dependent on growth factors to proliferate and survive.[112] For example, IL-3 growth factor regulates proliferation, differentiation and survival of hematopoietic cells by activating intracellular signaling pathways including Ras, MAPK, PI3K-Akt, and JAK-STAT. However in CML, BCR-ABL transforms hematopoietic cells and leads to IL-3 independence in vitro.[113] Hence, $BCR\text{-}ABL^+$ quiescent CML cells proliferation can be initiated without any added growth factor, while this is not possible for normal quiescent hematopoietic cells. Interestingly, BCR-ABL also promotes autocrine secretion of IL-3 in immature CML cells.[114] This aberrant autocrine production may confer a proliferative advantage to immature CML cells compared to normal ones. BCR-ABL can also replace growth factors signaling and drive the G_1 to S cell-cycle phase transition. Thus, this implies that BCR-ABL is able to activate mitogenic signaling pathways. For example, BCR-ABL has been demonstrated to activate Rat sarcoma (Ras) signaling, leading to the subsequent activation of Raf, MEK and ERK, and to an increase of cell proliferation.[115] This process plays a central role in the pathogenesis of CML as impairment of Ras signaling inhibits proliferation of primary CML cells and $BCR\text{-}ABL^+$ cell lines.[116]

In a physiological context, HSCs homeostasis is controlled by their interactions with their BM microenvironment. Indeed, the proliferation of normal hematopoietic progenitors is significantly inhibited when these cells are cultured in contact with BM stromal cells or in adhesion to fibronectin, suggesting that integrin-dependent adhesion inhibits normal

progenitor cell proliferation.[39] However in CML, BCR-ABL was shown to alter integrin-mediated adhesion of CML cells to their BM microenvironment, leading to an abnormal circulation of immature CML progenitors in the peripheral blood.[117] Thus, the abnormal expansion of CML progenitors, their growth advantages over existing normal progenitors and their evasion from the BM may be related to their inability to integrate and to transduce negative signals from integrin-mediated adhesion to the BM microenvironment.[117] However, this is controversial as in some cases, BCR-ABL-transduced hematopoietic cells displayed increased adhesive capabilities to their environment.[118]

Interestingly, in transformed cells, BCR-ABL has also been demonstrated to bind actin filaments (F-actin), a major determinant of cell mechanical behavior, and to induce its redistribution into punctate, juxtanuclear aggregates. By designing BCR-ABL mutants unable to bind F-actin but with an active kinase domain, it was demonstrated that the binding to F-actin seems to be involved in the transforming ability of BCR-ABL.[108]

To summarize, BCR-ABL kinase expression up-regulates cell proliferation by controlling cell-cycle, decreases apoptosis, increases cytokine-independent growth, decreases adhesion to the bone marrow stroma, and produces cytoskeletal abnormalities. As a result, BCR-ABL expression alone is recognized as the driving force of leukemogenesis in CML.

V. Diagnosis

Approximately 50% of CML patients are being diagnosed before they have become symptomatic as part of a routine check-up that reveals an elevated white blood cell count. Thus, more than 90% of CML patients are being diagnosed during the chronic phase (CP) of the disease.[91] However, considering the variable but generally long phase that precedes CP-CML diagnosis in different individuals, this suggests a high variability in the precise stage of the disease when the diagnosis is made. This bear important consequence as it affects how biological data are analyzed and how the results of clinical trials are interpreted. When symptoms are present they are usually non-specific and gradual in onset. They may include fatigue due to a lack of red cells causing anemia, frequent infections and slow healing due to a lack of normal white blood cells, increased sweating and unusual bleeding or bruising from various sites due to a very low platelet count, loss of appetite and weight loss. If the spleen is enlarged there may be abdominal pain or discomfort and a feeling of fullness when eating. An enlarged spleen can be observed during physical examination of CML patients but has become less common because of earlier diagnosis. The diagnosis must be confirmed by cytogenetic analysis to detect the Philadelphia chromosome and/or the BCR-ABL

rearrangement in peripheral blood or bone marrow cells.[91] In almost 5% of the cases, a Ph chromosome cannot be detected, and confirmation of diagnosis rests on molecular genetic methods, like fluorescent in situ hybridization (FISH) or reverse transcriptase-polymerase chain reaction (RT-PCR).

VI. Prognosis

In solid tumors, staging and treatment planning are usually based on the tumor size and the spreading of tumor cells from their original site. However this form of staging is not used in CML because the disease is typically widespread at the time of diagnosis. Thus, different scores (Sokal, Hasford, EUTOS) have been devised to help physicians predict which patients are more likely to have progressive disease. Among them, the Sokal score is the most commonly used, and derives from a multivariate survival analysis of 813 CP-CML patients at diagnosis.[119] Spleen size, platelet count and percentage of blast cells in the blood must be measured and performed before any treatment. The score, which is a hazard ratio, is calculated using the following formula: exp (0.0116 x (age [years] – 43.4)) + (0.0345 x (spleen size [cm] – 7.51) + (0.188 x ((platelets [109/L]/700)$^{\wedge 2}$ – 0.563)) + (0.0887 x (blasts [%] – 2.10)). Three risk groups have been determined: low-risk (Sokal score < 0.8, 39% of patients), intermediate-risk (0.8< Sokal score <1.2, 38% of patients) and high-risk (Sokal score > 1.2, 23% of patients). Although prognostic scores have been established before tyrosine kinase inhibitors (TKIs) development, they still allow the discrimination of risk groups with different prognosis, response rate, progression-free survival and overall survival, even for patients treated with TKIs. Before the clinical use of TKIs, the median survival time for CML patients was comprised between 3 to 5 years from time of diagnosis. TKIs development led to very significant improvements in patient's survival and quality of life. A follow-up of 832 patients who achieved a stable cytogenetic response under Imatinib (TKI actually used for CML first-line therapy) treatment found an overall survival rate of 95.2% after 8 years, which is similar to the rate in the general population. Less than 1% of patients died because of leukemia progression, leading to a general agreement that this drug will prevent disease progression in the majority of patients.[120] However, the minority of patients who were diagnosed in blast crisis gained much less benefit from TKIs introduction. The degree and timing of hematologic, cytogenetic and molecular responses provide very important prognostic information as time-dependent variables. In particular, the prognostic importance of complete cytogenetic response (CCyR) has been confirmed.

VII. Disease evolution

Clinically, the natural course of the disease includes three distinct phases. Without treatment, leukemic cells tend to acquire additional genetic abnormalities over time, leading to disease progression from an initial chronic phase (CP) to an accelerated phase (AP) and to an inexorable fatal blast crisis (BC). The acquisition of secondary genetic changes is thought to be the consequence of BCR-ABL inhibitory effects on cellular DNA reparation mechanisms, thus leading to genomic instability.[121] For example, loss of p53 function might be important for CML disease progression, as p53 is inactivated in almost 30% of CML blast-crisis cases.[122] The initial phase is characterized by a progressive myeloid expansion, with accumulation of myeloid progenitors and mature granulocytes in the bone marrow (BM) and peripheral blood. During this phase, CML cells have been shown to be functionally heterogeneous and capable of maintaining a hierarchical organization caricaturing normal hematopoiesis, with only a fraction of the cells being actually responsible for disease maintenance and propagation, thus behaving as leukemia-initiating stem cells. Upon acquisition of secondary mutations, this chronic phase evolves through an accelerated phase into an acute leukemia-like blast crisis involving either myeloid (about half of the blast crisis), B lymphoid (about a quarter of the blast crisis) or myeloid/lymphoid biphenotypic cells (about a quarter of the blast crisis).[122] Ultimately, this leads to a failure of the blast phase cells to differentiate and hence to a rapid accumulation of non-functional cells in the bone marrow and peripheral blood. Hence, the blast crisis is characterized by the presence of at least 30% blast cells in the bone marrow or circulating blood. The blast crisis, now increasingly rare but still encountered, is usually associated with significant symptoms. Clinically, BC resembles acute leukemia, with a poor prognosis and often resistance to therapy. The median survival time for myeloid and lymphoid BC-CML is 4 months to 5 months and 12 months, respectively.[123]

VIII.Treatment

1. Conventional therapies

The history of CML treatment begins in 1878 with the use of arsenic to decrease the levels of white blood cells in patients. The early 1900s was then marked by the discovery of X-rays and the clinical use of radiotherapy. Whole body or splenic radiation gradually became the reference treatment for this disease until the 1950s, but was only palliative, as it did not improve overall survival of the patients.[86]

From the 1950s, a cell-cycle non-specific alkylating chemotherapeutic agent called

busulfan was employed, and was able to maintain partial hematologic response in most CML patients, however with serious side-effects. Indeed, chronic use of busulfan has been associated with a syndrome that stimulates adrenal insufficiency, manifested by skin pigmentation, weakness, fever, and diarrhea, or with pulmonary fibrosis. Prolonged aplasia of the marrow can also occur with busulfan.[124]

Hydroxyurea, an inhibitor of DNA synthesis and cell-cycle, was introduced to treat CML in the 1970s. Hydroxyurea is less toxic than busulfan and sustains the chronic phase of the disease for a longer time. The major side effect of hydroxyurea is that it causes suppression of hematopoiesis that is reversible. The median survival of CML patients treated with hydroxyurea is about 5 years, and is longer than for patients treated with busulfan, which is about 3,75 years. Unfortunately, both busulfan and hydroxyurea treatments simply keep the tumor size under control without affecting its subsequent course. Under these treatment conditions, progression from the chronic phase to the fatal blast phase is inevitable.[125]

In the early 1980s, stem cell transplantation (SCT) was developed as a revolutionary therapy and remains the only curative treatment for CML patients. SCT involves the introduction of donor (allogeneic) or the patient's own treated (autologous) HSC into the patient after treatment with high doses of chemotherapy and/or radiation to destroy all bone marrow cells (cells used for autologous transplant are withdrawn prior to this step), in the hope that they will repopulate the patient's marrow and replace the CML LSC.[126] However, its relevance in the post-Imatinib era is debated. Indeed, only about 20% of CML patients are suitable for allogeneic SCT due to age of the patient, fitness and donor availability. Moreover, SCT can also be responsible of transplant-related mortality that ranges from 20% in low-risk CML patients to 73% in high-risk patients.[126] The high mortality rate is usually caused by graft-versus-host disease and opportunistic infections. Yet, it remains an important option, particularly for younger patients with HLA (Human leukocyte antigen) identical siblings with the hope of cure, or patients who are intolerant or resistant to TKIs.

In the 1980s, Interferon alpha (IFNα) became the first line therapy of CML patients who were not eligible for allogeneic SCT. It was the first treatment that consistently produced durable cytogenetic remissions in 10% to 20% of CML patients.[86] IFNα is a cytokine produced by leukocytes and mainly involved in innate immune response. However there are early side effects (fever, fatique, anorexia, nausea…) as well as later side effects (apathy, thrombocytopenia, renal or cardiac dysfunction…). Combination of hydroxyurea and interferon led to 73% of hematologic response rate and 58% of cytogenetic remission rate in chronic phase CML patients.[86] While IFNα monotherapy is no longer recommended because of TKIs development, its combination with Imatinib is currently being tested in phase III prospective studies.

2. Tyrosine Kinase Inhibitors

As the understanding of the molecular biology of CML progressed further, BCR-ABL became an attractive therapeutic target for CML. In the mid-1990s, Imatinib (also known as STI-571 or Gleevec), a phenylaminopyrimide derivative with potent tyrosine kinase inhibitory activity and selectivity for Abl, c-Kit, and platelet-derived growth factor receptor (PDGF-R) was developed by random screen of a large number of synthesized ATP-competitive compounds. Indeed, Imatinib binds to a pocket of the catalytic domain of the Abl tyrosine kinase and competitively inhibits the binding of adenosine triphosphate (ATP), thereby resulting in inhibition of autophosphorylation and inhibition of substrate phosphorylation (Figure 11).[127] The activation loop in the enzymatic site of Abl controls its kinase activity by switching between active and inactive conformations. Imatinib binds to Abl only when it is in the inactive state and prevents it from changing into the active conformation.[127] Imatinib inhibits proliferation and causes apoptosis in BCR-ABL$^+$ cell lines and primary cells both *in-vitro* and *in-vivo*, with very low toxicity for BCR-ABL$^-$ cells, demonstrating the specificity of this drug against BCR-ABL$^+$ cells.[128–130] This first tyrosine kinase inhibitor (TKI), competitively inhibiting the BCR-ABL kinase, was approved based on the results of the International Randomized Study of Interferon-α versus STI571 (IRIS) trial showing that at 18 months, 76.2% of the patients achieved complete cytogenetic response (CCyR) and 96.7% were free from disease progression.[131] The update of the IRIS study has confirmed and extended the earlier results, reporting a progression-free survival of 84% and an overall survival of 88% after 6 years.[132] Until recently, Imatinib has become the gold standard for CP-CML care. Since the advent of this molecule (Figure 12A), CML has become the first cancer in which a standard medical treatment may give to the patient a normal life expectancy.

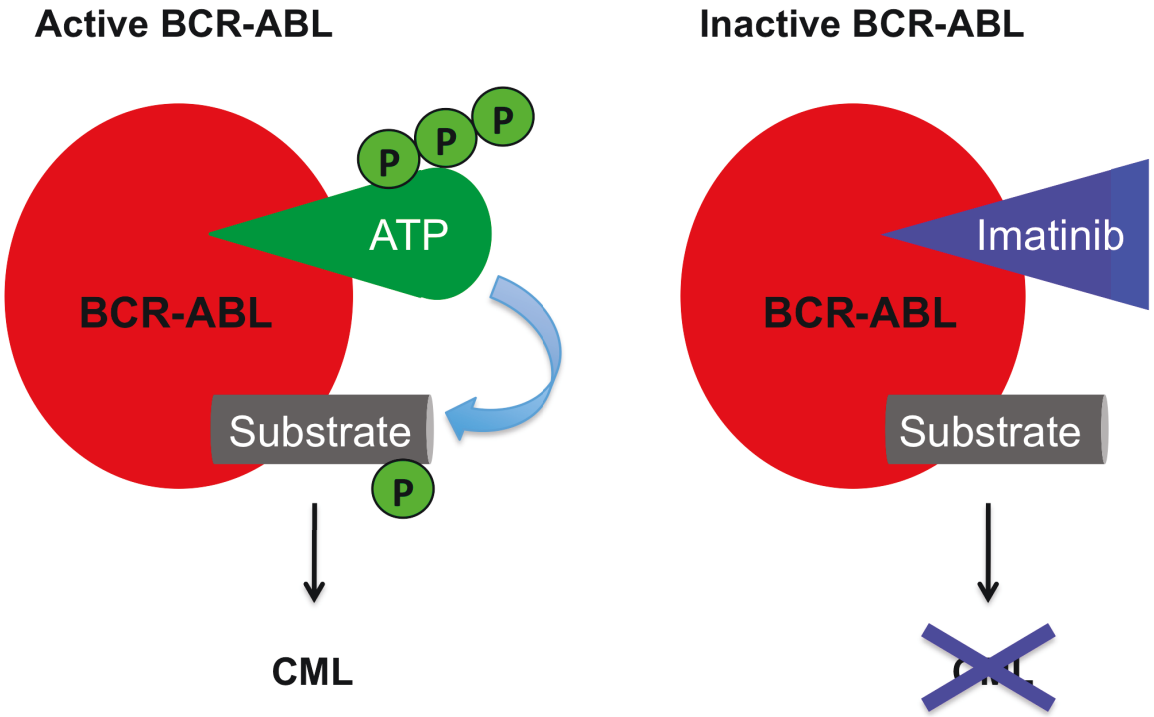

Figure 11 : Mechanism of action of Imatinib on BCR-ABL. ATP binding is essential for BCR-ABL to phosphorylate substrates and subsequently activate downstream pathways that promote cell survival and proliferation leading to CML. Imatinib can bind to the inactive state of BCR-ABL at its ATP binding site, and inhibit the phosphorylation of substrates that would activate downstream signaling and lead to CML.

The response to Imatinib may fall into three categories, namely optimal, suboptimal and failure. In case of 'failure', second-line treatment is based on second-generation TKIs dependent on the identified *Bcr-Abl* mutation, namely Nilotinib and Dasatinib.[133] About 50% of CP-CML patients resistant or intolerant to Imatinib achieve a CCyR with either agent, but both agents are ineffective in the case of a T315I BCR-ABL kinase domain (KD) mutation. The response to either agent is usually rapid, and within 6 months it may be possible to decide to continue with the second-generation TKIs or to move to allogeneic SCT, if the patient is eligible. In the case of suboptimal response to Imatinib, which frequently represents a transitory state, the best treatment option is still a matter of investigation. The patients can be continued on Imatinib, same dose or higher dose, but are also eligible for a trial with second-generation TKIs. Once a patient has progressed to advanced phase, further treatment depends on prior therapy, and may include other TKIs, different from those used in chronic phase, experimental targeted agents or cytotoxic chemotherapy, always with the purpose of performing an allogeneic SCT, whenever it may be possible.

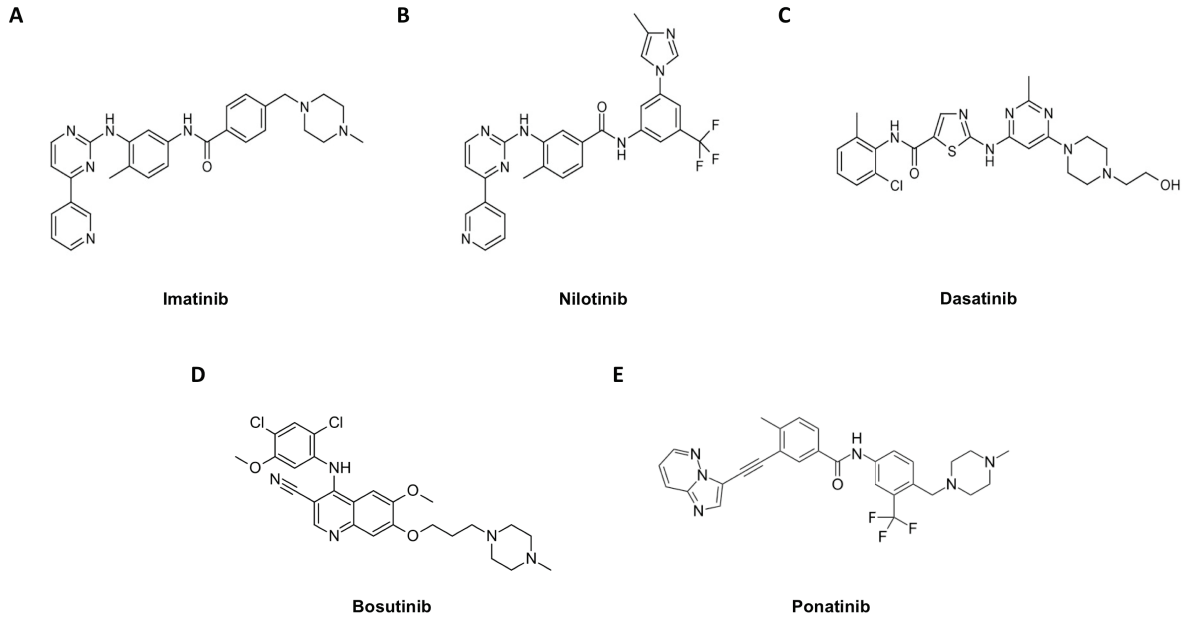

Figure 12 : Tyrosine kinase inhibitors molecular structure. (A) Imatinib structure. A high-throughput screening of chemical libraries at Novartis was performed to identify a starting molecule, which was called pyrimidine A. This compound served as a lead compound and was then tested and modified to develop Imatinib. With a replacement of the imidazole group with a benzamido group, the compound's specificity increased while its activity as a kinase inhibitor remained the same. Subsequently, introducing a methyl subtituent ortho to the pyrimidinyl-amino group enhanced the potency. (B) Nilotinib structure. Nilotinib is a phenylamino-pyrimidine derivative that is structurally related to Imatinib. (C) Dasatinib structure. Dasatinib is a thiazolylaminopyrimidine developed as the hydrochloride salt. (D) Bosutinib structure. Bosutinib is a 4-anilinoquinoline-3-carbonitrile inhibitor. (E) Ponatinib structure. Ponatinib is a N-phenylbenzamides derivated compound.

Second generation TKIs (Nilotinib, Dasatinib, Bosutinib) have emerged to target BCR-ABL in order to overcome Imatinib resistance. Nilotinib (also known as AMN107 or Tasigna) is a rationally designed specific inhibitor of Abl tyrosine kinase (Figure 12B). Apart from Abl kinase, Nilotinib also inhibits the activity of c-kit and PDGF-R.[134] Nilotinib was designed to have higher binding affinity than Imatinib to the ATP binding site on the kinase domain of BCR-ABL.[135] Just like Imatinib, Nilotinib binds to the inactive conformation of Abl kinase, and blocks its tyrosine kinase activity. Nilotinib inhibits 32 out of 33 Imatinib-resistant BCR-ABL mutants except the T315I mutation.[135,136] Nilotinib exhibits about 20 times more potency than Imatinib in Imatinib sensitive and insensitive BCR-ABL$^+$ cell lines. Although Nilotinib is much more effective than Imatinib in BCR-ABL$^+$ cell lines, Nilotinib and Imatinib are equally potent in human primary CD34$^+$ CML cells, and only have anti-proliferative effect instead of eradicating these cells.[137] Nonetheless, clinical studies have demonstrated the effectiveness and safety of Nilotinib in Imatinib resistant or intolerant CML patients,[138] leading to the approval of Nilotinib by FDA for treating adult BCR-ABL$^+$ CP- and AP-CML patients who are resistant to or intolerant of prior therapy including Imatinib, and subsequently newly

diagnosed adult BCR-ABL$^+$ CP-CML patients.[139]

Dasatinib (also known as BMS 354825 or Sprycel) is a dual Src/Abl tyrosine kinase inhibitor (Figure 12C). It also targets c-kit and PDGF-R.[140] Dasatinib is 325-fold more potent than Imatinib in cells expressing wild-type BCR-ABL protein.[138] Almost all BCR-ABL kinase domain mutations can be inhibited by Dasatinib except once again the T315I mutation. Dasatinib binds to both active and inactive conformations of Abl kinase, rendering it much higher binding affinity than Imatinib.[141] Clinical trials demonstrated the efficacy of Dasatinib in all phases of Imatinib resistant or intolerant CML patients, and it is well tolerated.[142] Dasatinib and Nilotinib have equivalent response rates with a complete hematologic response rate of about 87% and 92% respectively in CP-CML patients.[143] Dasatinib was approved by FDA for treatment of adult BCR-ABL$^+$ CML and ALL patients who are resistant to or intolerant of prior therapy (such as Imatinib), as well as newly diagnosed adult BCR-ABL$^+$ CP-CML patients. However, Dasatinib is still not able to eliminate the primitive quiescent CML stem cell population.[144] For this reason, although both Nilotinib and Dasatinib have improved effects in suppressing CML than Imatinib, they are still not able to cure CML.

Bosutinib (also known as Bosulif or SKI-606) is a dual Src/Abl TKI with a more potent *in-vitro* inhibitory activity against BCR-ABL than Imatinib and a minimal inhibitory activity against c-kit and PDGF-R, which are potentially associated with some toxicities reported for other TKIs (Figure 12D).[145] While Bosutinib seems more efficient than Imatinib to induce major molecular response by 24 months (59% versus 49%), more Bosutinib-treated patients suffered adverse events and had to stop the treatment compared to Imatinib-treated patients (25% versus 9%).[145] Bosutinib is ineffective against the T315I mutation. Bosutinib was recently approved by the FDA for the treatment of adult BCR-ABL$^+$ CML with resistance, or intolerance to prior therapy.

Several third generation TKIs were designed to target T315I, but only a few agents have shown clinical activity. Ponatinib (also known as Iclusig or AP24534) is a third generation TKI that seems to be the only effective drug in patients with T315I mutation (Figure 12E). Based on positive phase 1 and phase 2 trials, Ponatinib was approved for the second-line treatment of CML and BCR-ABL$^+$ ALL in 2012.[146] However, due to severe vascular adverse events such as blood clots formation and narrowing of blood vessels, its use in clinic is still debated.

IX. Monitoring treatment results

1. Hematologic response

Monitoring the patient to see how they respond to treatment is very important because this will allow to accurately assess the treatment efficiency and to provide an early indication of emerging drug resistance. First, the hematologic response is usually checked about 3 months after the beginning of TKI treatment by blood cell count. When blood cell counts return to normal (leukocyte count < 10×10^9/L), no immature cells are observed in the blood, and the spleen has returned to normal size, it is called a complete hematologic response (CHR) (Figure 13).[133] A partial hematologic response is similar to this, but not all of the above conditions are met.

2. Cytogenetic response

Then, karyotyping of FISH analysis can also be performed to monitor the percentage of BCR-ABL$^+$ cells among the total bone marrow cells. A complete cytogenetic response (CCyR) occurs when no cells with the Philadelphia chromosome can be found in the bone marrow (Figure 13). A partial cytogenetic response (PCyR) occurs when less than 35% of cells still have the Philadelphia chromosome. A minor cytogenetic response occurs when 35% to 90% of cells still have the Philadelphia chromosome.[133]

3. Molecular response

Finally, the blood or the bone marrow may also be checked by real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) to measure the amount of *Bcr-Abl* transcripts. A major molecular response (MMR) means that the amount of *Bcr-Abl* mRNA detected is very low (≥ 3 log reduction compared to the standardized baseline) (Figure 13).[147] Achieving an MMR is an important predictor of progression-free survival in CML.[148] A complete molecular response (CMR) means that the *Bcr-Abl* transcripts are not detected, or at extremely low levels (≥ 4.5 log reduction compared to the standardized baseline). This is especially important since the use of second-generation TKIs (Dasatinib and Nilotinib) as frontline therapy for CP-CML, where higher response rates are achieved at earlier time points when compared with standard-dose Imatinib therapy.[133] However, the results of treatment monitoring have to be considered with caution, as poor adherence to TKI treatment has recently been described as the principal factor contributing to the loss of cytogenetic responses and treatment failure in patients on long-term therapy.[149]

Type of testing	Technique	Response	Response criteria
Hematologic	Blood cell count using blood sample	Complete hematologic response (CHR)	Complete normalization of peripheral blood counts
Cytogenetic	Karyotyping or FISH analysis using bone marrow aspirate	Minor cytogenetic response (MCyR)	35% - 90% Ph+ cells or metaphases
		Partial cytogenetic response (PCyR)	<35% Ph+ cells or metaphases
		Complete cytogenetic response (CCyR)	0% Ph+ cells or metaphases
Molecular	qRT-PCR using blood sample or bone marrow aspirate	Major molecular response (MMR)	≥3 log reduction of BCR-ABL mRNA compared to the standardized baseline
		Complete molecular response (CMR)	No detectable BCR-ABL mRNA by qRT-PCR using an assay with a sensitivity of ≥4.5 log below the standardized baseline

Figure 13 : Monitoring TKIs treatment efficiency in CML patients. Hematologic response is the first objective to attain when treating patients and corresponds to the normalization of blood cell counts. Then, the cytogenetic response will display the percentage of BCR-ABL$^+$ cells among the total bone marrow cells. Finally, the molecular response is the most sensitive assay, as the qRT-PCR technique will allow the detection of very low *Bcr-Abl* transcripts levels, the aim being to attain the lowest *Bcr-Abl* level as possible.

X. Mechanisms of TKI resistance

1. Definition

Despite the positive results obtained in previous studies, approximately 30% of CML patients treated with Imatinib do not achieve a complete cytogenetic response, while others have drug resistance or cannot tolerate drug-related toxicities.[150] Resistance is defined on the basis of its time of onset. Primary resistance is a failure to achieve a significant cytogenetic response, whereas secondary or acquired resistance is the progressive reappearance of the leukemic clone after an initial response to the drug (Figure 14).[151,152] Primary resistance can be further divided into primary hematologic resistance, which occurs in 2% to 4% of cases who fail to normalize peripheral counts 6 months after treatment, or primary cytogenetic resistance, which is more common and occurs in approximately 15% to 25% of patients who fail to achieve any level of cytogenetic response (CyR) at 6 months, a major CyR (MCyR) at 12 months or a CCyR at 18 months.[153] Secondary resistance occurs in those who have previously achieved and subsequently lost their response to the treatment (Figure 14). Complete elimination of CML clones has rarely been achieved by TKIs because of the development of a variety of cell-intrinsic and cell-extrinsic protective mechanisms. Extrinsic mechanisms are supported by the interplay between LSCs and their protective bone marrow microenvironment, the so-called “leukemic niche” (Figure 14). Intrinsic resistance will be further divided between BCR-ABL dependent and independent

mechanisms (Figure 14). BCR-ABL dependent mechanisms include increased BCR-ABL expression and point mutations within the BCR-ABL kinase domain. BCR-ABL independent mechanisms are not so well understood and include mostly defects in drug transport in and out of the leukemic cells and activation of secondary oncogenic pathways.[154]

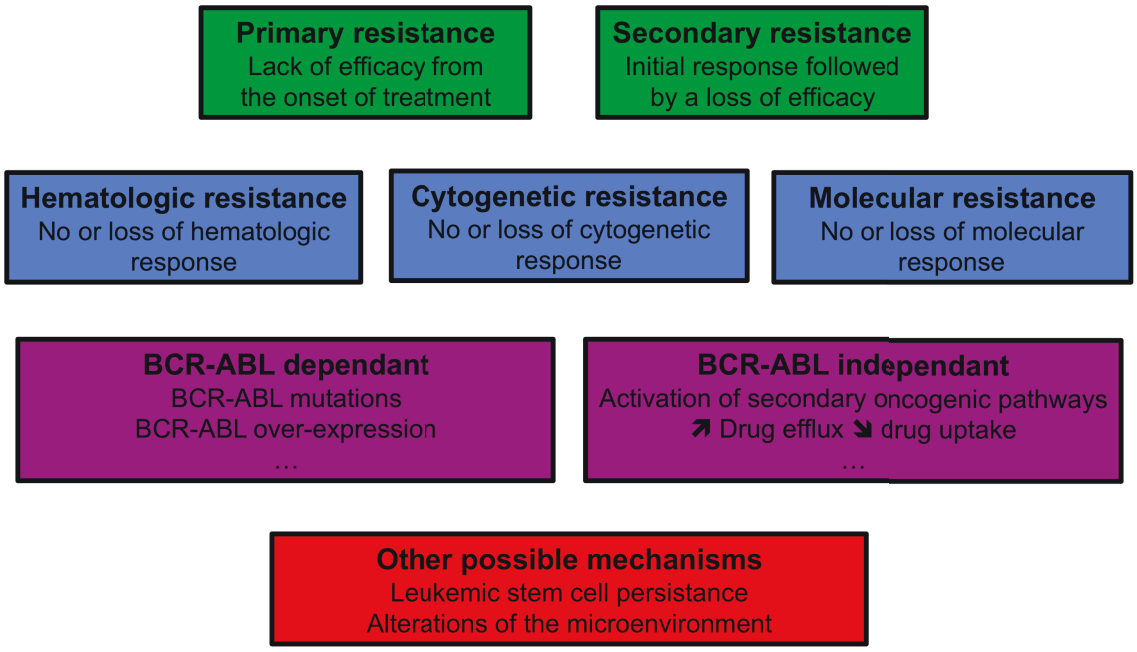

Figure 14 : Resistance to tyrosine kinase inhibitors treatment: description and mechanisms. Resistance to TKIs treatment can be primary or secondary, defined as hematologic, cytogenetic or molecular, and can involve BCR-ABL dependant or independent mechanisms. The most frequent mechanism involved in TKIs resistance is the development of point mutations in BCR-ABL kinase domain as well as BCR-ABL over-expression. Other mechanisms have also been identified such as activation of BCR-ABL independent signaling pathways, increase of drug efflux or decrease of drug uptake. But some resistance mechanisms are still unknown and could involve LSCs that may survive TKIs treatment, for example through entry into a quiescent state or the alteration of their microenvironment.

2. BCR-ABL mutations

The development of point mutations in the kinase domain is the most frequent mechanism of acquired resistance as it is found in about 40% to 50% of cases.[154] These mutations are not induced by the drug, but rather, arise through a process where rare pre-existing mutant clones are selected due to their capacity to survive and expand in the presence of the drug, thus gradually outgrowing drug sensitive cells. More than 100 different point mutations leading to the substitution of approximately 50 amino-acids in the kinase domain have been isolated from resistant CML patients so far, and this number is likely to increase with the development of more sensitive methods of detection.[154] The substitution of the amino acid threonine with isoleucine at position 315 of the ABL protein, or T315I, was the

first and most frequent mutation observed in resistant patients.[155] This is the most problematic mutation to deal with in clinic because of its resistance to all approved BCR-ABL inhibitors, prior to Ponatinib. Designed to be effective against T315I mutations, this third generation TKI lead to 82% of achieved major cytogenetic response (MCyR) and 40% of achieved MMR in patients with the T315I mutation in a phase I trial.[156] However, significant adverse effects were observed in patients treated with this drug compared to other TKIs, including fatigue, nausea, thrombocytopenia, rash, pancreatitis, arterial thrombotic events and hepatotoxicity.

3. BCR-ABL over-epression

BCR-ABL over-expression has also been described as a resistance mechanism in CML patients, by increasing the amount of target protein needed to be inhibited by the therapeutic dose of Imatinib. Increased BCR-ABL levels due to amplification of the BCR-ABL gene was first described in resistant CML cell lines generated by exposure to gradually increased doses of Imatinib.[157] Cells expressing high levels of BCR-ABL are far less sensitive to Imatinib and will also more rapidly develop Imatinib resistant mutant subclones compared to cells with low BCR-ABL expression levels.[155] This is thought to be related to an increased genomic instability induced by high levels of BCR-ABL in some clones, thus leading to a higher propensity to develop mutations anywhere in the genome, including in the BCR-ABL kinase domain.[121,158,159]

4. Drug efflux increase

Multidrug resistance (MDR) is a well-known phenomenon involved in mammalian cells resistance to a number of anticancer agents following drug exposure.[160] This is due to the presence of efflux pumps, evolved defense mechanisms present in animals, fungi and bacteria to protect them against harmful substances by excluding them from the cells. In most cases, the resistance is mediated by an increased expression at the cell surface of the Pglycoprotein (Pgp) encoded by the MDR1 (ABCB1) gene. Pgp is an ATP-dependent efflux pump with broad substrate specificity, which primarily reduces intracellular drug concentrations, leading to the failure of effective levels of the drug to reach its target.[161] Imatinib and other TKIs have been demonstrated to be substrates of Pgp and the intracellular levels of Imatinib were shown to be significantly lower in Pgp-expressing cells.[162] Pgp over-expression was observed in an Imatinib-resistant CML cell line, and was demonstrated to confer Imatinib resistance. However, clinical studies have failed to find a similar association, and inhibition of MDR1 does not seem to enhance the effect of Imatinib against BCR-ABL activity.[163] Thus, the significance of Pgp expression in Imatinib resistance

has yet to be fully clarified. Another drug efflux pump over-expressed in many human tumors, the breast cancer resistance protein (BRCP) encoded by the *Abcg2* gene, was also found to be functionally expressed in CML LSC and has been implicated in TKIs resistance. Nevertheless, a recent study showed that ABCB1[163] and ABCG2[164] seem to have a minimal functional role in the transport of Imatinib in primary CML $CD34^+$ cells.

5. Drug uptake decrease

The failure of TKIs to effectively inhibit leukemic cells could also result from impairment of drug uptake by influx pumps, so that it never reaches its effective intracellular concentration. It has been reported that Imatinib is actively transported into cells by organic cation transporter 1 (OCT1), a member of the solute carrier transporters encoded by the *Slc22* gene family, and that OCT1 inhibition decreases the intracellular concentration of Imatinib.[165] OCT1 expression has been correlated with intracellular Imatinib uptake or activity in CML cells and may thus determine the therapeutic outcome in patients. Indeed, the OCT1 gene is expressed at significantly higher levels in mononuclear cells (MNC) from patients who achieved a complete cytogenetic response (CCyR) to Imatinib than from those who were more than 65% Ph chromosome-positive after 10 months of treatment.[165,166] Interestingly, low OCT1 activity has been demonstrated to reduce Imatinib uptake in $CD34^+$ CML cells.[167] However, neither Nilotinib nor Dasatinib cellular uptake was significantly affected by OCT1 activity.[165,168]

6. Activation of BCR-ABL independent signaling pathways

Another resistance mechanism observed in CML cells is the activation of BCR-ABL independent signaling pathways that will be sufficient to cause disease progression even when BCR-ABL activity is inhibited by TKIs treatment. Such activated secondary signaling pathways have been identified, like Lyn kinase, Ras/Raf/MEK kinase, Fyn/Erk or STAT signaling.[150] For example, Lyn, a Src family kinase expressed on myeloid cells[169] and activated in BCR-ABL expressing cells has been demonstrated to be over-expressed and activated in Imatinib resistant CML cell lines.[170,171] Interestingly, Lyn suppression by a Src kinase inhibitor resulted in reduced proliferation and survival of the Imatinib resistant but not the sensitive cell line. Lyn over-expression and BCR-ABL independent activation were described in CML cells from Imatinib resistant patients. Since BCR-ABL binding leads to Lyn activation and, in cells with high Lyn expression or activation,[172] inhibition of BCR-ABL kinase by Imatinib is not sufficient to abolish BCR-ABL mediated signaling completely, it is possible that both BCR-ABL and Lyn inhibition are needed to prevent this resistance to Imatinib.[173]

7. Autophagy

Autophagy is another possible mechanism for CML cells to survive under TKI treatment. Autophagy is a pathway that degrades proteins or organelles to maintain cellular homeostasis, and also generates energy under starvation or other cellular stress.[174] Imatinib has been demonstrated to induce autophagy in CML cell lines as well as in primary CML cells.[175] Blockage of autophagy with chloroquine increases Imatinib induced apoptosis in CML cells.[174] Interestingly, this phenomenon was also observed in CML progenitor and stem cells.[175] This indicates that autophagy may serve as a survival mechanism for CML cells under TKI treatment,[176] and that autophagy inhibition by drugs may be used to potentiate TKI-induced apoptosis in primitive CML cells.[177]

Other mechanisms have also been described, like low serum drug concentration, or Imatinib sequestration by increased serum protein α1 acid glycoprotein, which binds Imatinib and impairs subsequent binding to BCR-ABL.[178] Elevated transcript levels of prostaglandin-endoperoxide synthase 1/cyclooxygenase 1, which encodes an enzyme that metabolizes Imatinib, has also been associated with primary resistance.[179] In some cases, the resistance mechanisms have yet to be identified, and one of the hypothesis that could explain those mechanisms is the existence of LSCs, that could survive to drug treatment due to their intrinsic specific properties and to their interactions within their microenvironment.

XI. CML LSC persistence and resistance

1. LSC persistence

Although TKIs advent has proved remarkably effective to improve CML patients care, even patients in complete cytogenetic remission (CCyR) still harbor minimal residual disease, therefore sustaining detectable disease over the years. Indeed, none of the therapeutic agents available to date seem able to eradicate some discrete undifferentiated BCR-ABL$^+$ cells. Recent evidences suggest that BCR-ABL$^+$ LSCs are insensitive to TKIs treatment and can regenerate the disease upon drug discontinuation in 61% of cases, thus requiring continued treatment.[180] These cells may also serve as a reservoir for additional oncogenic events leading in some cases to disease progression and secondary resistance mechanisms. Therefore, the degree to which the levels of residual disease can be reduced during therapy has become an increasingly recognized objective of CML clinical trials, assuming that reduction of this tumor load is of prognostic relevance.

2. Resistance and oncogene independence

CML treatment relies on the concept of oncogene addiction, postulating that some tumours depend on one single dominant oncogene for growth and survival, so that inhibition of this specific oncogene is sufficient to block the tumorigenic phenotype.[181] In CML, Imatinib treatment has proved particularly effective to repress BCR-ABL tyrosine kinase activity leading to inhibition of cell proliferation and apoptosis. However, recent *in-vitro* and *in-vivo* results demonstrated that CML LSCs might not be "oncogene addicted".[181] This was demonstrated by serial transplantation of CML LSCs after abrogation of BCR-ABL expression using a tet-off inducible transgenic mouse model of CML-like disease in which p210-BCR-ABL expression is targeted to murine BM stem and progenitor cells.[181] Indeed, after serial transplantations, donor-derived CML LSCs in which BCR-ABL expression had been induced and subsequently shut off were able to persist *in-vivo* and reinitiate leukemia in secondary recipients upon BCR-ABL re-expression. The results of this study are particularly striking, because they mean that as normal HSCs, CML LSCs (or at least some of them) do not depend on BCR-ABL expression for their survival. However, these cells still harbor the BCR-ABL oncogene, and are able to reconstitute the disease upon TKIs treatment arrest or acquisition of secondary mutations (Figure 15).[181] But more importantly, these LSCs also display normal HSCs intrinsic properties such as high DNA repair ability, quiescence potential, over-expression of anti-apoptotic proteins, of drug efflux transporters and of detoxifying enzymes. These properties allow normal HSC to persist throughout an individual's entire life to maintain a physiological hematopoiesis, while in LSCs the consequences will be an increased resistance to drug treatment. Thus, it is crucial to understand the specific mechanisms of LSCs resistance and to develop new therapies to efficiently eliminate these cells without damaging normal HSCs.

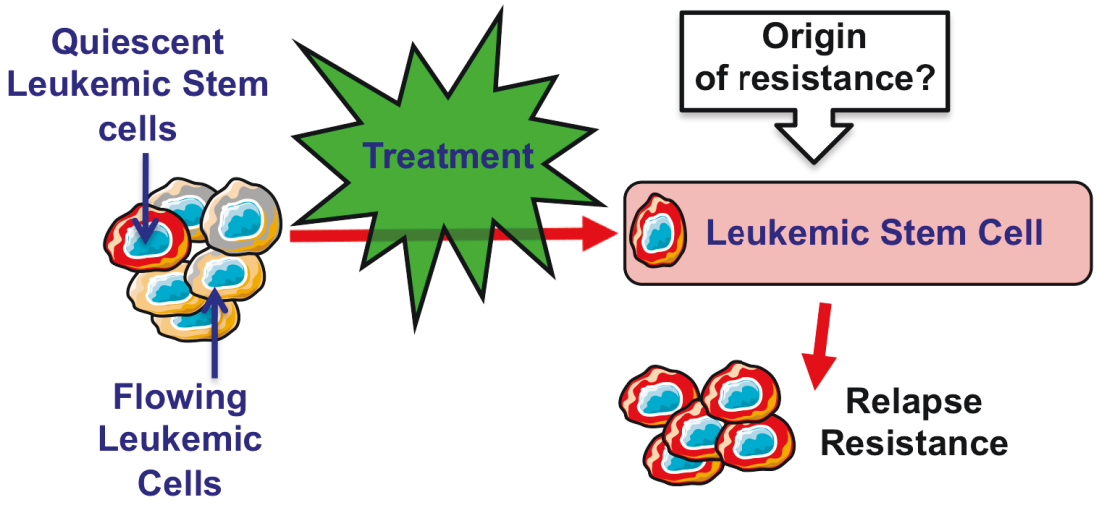

Figure 15 : Resistance to treatment, the leukemic stem cells hypothesis. Unlike differentiated leukemic cells, LSCs are suspected to survive to TKIs treatment, probably by their capability to enter into a quiescent state. Then, these cells could lead to relapses in the patients, after arrest of the treatment or by different despite unknown mechanisms, probably involving the acquisition of secondary alterations.

However, LSCs do not only rely on their intrinsic mechanical properties for their survival. Indeed, like normal HSCs, CML LSCs are also located in the BM microenvironment that produces different signals participating in SC fate and survival.

XII. Microenvironment involvement

1. Quiescence

Although less well studied, there is some evidence that LSCs resistance to TKIs treatment may also be supported by their bone marrow microenvironment. *In-vitro*[182] and *in-vivo*[183] studies have demonstrated that TKIs have an anti-proliferative effect on CML LSCs, causing them to enter reversible cell-cycle arrest and accumulate in a quiescent state. However, these cells are resistant to TKIs induced apoptosis even with intracellular levels of TKIs similar to those observed in more mature CML cells.[154] Thus, such cells may persist in a dormant state as a residual and highly resistant population of CML LSC with the capability to repopulate the leukemic clone, even in patients who are in complete molecular response with undetectable *Bcr-Abl* transcripts. Quiescent LSCs represent less than 1% of the total CML stem/progenitor cell population. Therefore, forcing these cells out of dormancy is required to eliminate them. Stimulating quiescent LSCs to enter the cell-cycle with granulocyte colony stimulating factor (G-CSF) reduces the overall non-cycling cell population *in-vitro* but, in

clinical practice, does not impact on disease outcome.[184] Recently the farnesyl transferase inhibitor BMS-214662 was found to kill quiescent CML progenitors and blast crisis CML cells *in-vitro* and its effect was enhanced when combined with either Imatinib or Dasatinib, making it a promising agent for clinical development.[185] Moreover, BCR-ABL specifically inhibits CXCR4, the receptor for SDF1, a chemokine produced by stromal cells that mediates the homing of $CD34^+$ progenitor cells to the bone marrow microenvironment.[186] Interestingly, Imatinib has recently been found to restore CXCR4 expression, which promotes the migration of CML cells to the BM.[187] CML cells homing is associated with G_0-G_1 cell-cycle arrest, inhibition of their proliferation, and enhanced survival in a quiescent state under TKIs treatment. Therefore, pharmacological inhibition of CXCR4 is expected to reverse this mechanism of primary resistance, a concept already demonstrated with the CXCR4 antagonist Plerixafor (also known as AMD3100).[188] Thus, combining inhibitors of CXCR4 and TKIs may represent a potential opportunity for future CML care.

2. Soluble factors

In a recent study, the authors prepared conditioned media from different human BM stromal cell lines, and then cultured CML cells in these media in presence or absence of TKIs. For some conditioned media but not for all, they observed an enhanced resistance of CML cell lines under TKIs treatment. They then determined the cytokines specifically produced in conditioned media favoring resistance, and identified several molecules signaling through JAK2/STATS pathway (G-CSF, GM-CSF, and IL-6).[189] Others molecules produced in the BM microenvironment have also been identified for their role in CML cells resistance to TKIs, such as Fibroblast growth factor 2 (FGF2). Indeed, it has been demonstrated that FGF2 signaling through FGF receptor 3/RAS/c-RAF/mitogen-activated protein kinase pathway induced K562 cell line growth in both short- and long-term assays under Imatinib treatment. Resistance could be overcome with Ponatinib, a TKI inhibitor that targets both BCR-ABL and FGF receptor.[190] Clinically, resistant CML patients without kinase domain mutations who responded to Ponatinib treatment displayed increased FGF2 levels in their bone marrow. Moreover, FGF2 levels in the bone marrow of CML patients decreased during Ponatinib response, further suggesting that FGF2-mediated resistance is interrupted by FGF receptor inhibition.[190]

3. Cell adhesion-mediated drug resistance

Although CML is characterized by a vast expansion of the malignant cell population that leave the BM microenvironment prematurely, some leukemic cells maintain their adhesion potential and can still be found within the BM. The BM microenvironment has been

demonstrated to efficiently protect leukemic cells against apoptosis induced by TKIs, cytotoxic drugs and gamma-irradiation. Indeed, CML cells in contact with stromal cells or extracellular matrix components such as fibronectin may acquire resistance to TKIs by a process known as cell adhesion-mediated drug resistance (CAM-DR).[191] Indeed, adhesion mediated by α5β1 integrin has been demonstrated to induce an increased drug resistance in CML cells, by inducing the degradation of the pro-apoptotic BCL-2 family member protein Bim.[192] However, the exact mechanisms involved are still not clearly identified, but may also involve other mechanisms such as cell-cycle arrest. Moreover, another study demonstrated that antibody-mediated targeting of the CD44 adhesion receptor could prevent trafficking of CML LSCs to the BM, decrease engraftment in immunodeficient mice and promote LSC differentiation.[193] These results are particularly interesting, as CD44 has been described as a CSC marker in different types of cancer, such as ovarian cancer, breast cancer or AML, and because of the development of very promising drugs targeting this receptor.[194] Due to the central role of cell interactions in HSCs and LSCs control by their microenvironment, more and more studies use co-culture systems to reproduce these interactions and to identify the functional consequences *in-vitro*. In a recent study, CML leukemic stem ($CD34^+$, $CD38^-$) and progenitor ($CD34^+$, $CD38^+$) cells co-cultured with human BM mesenchymal stromal cells, leading to a significant inhibition of CML cells apoptosis following TKIs exposure.[195] This was associated with the maintenance of colony-forming ability and engraftment potential in immunodeficient mice. Using transwell assays, they demonstrated that contact between the two cell populations was required to protect CML LSCs during TKI treatment. Further analysis then revealed the role of N-cadherin receptor activation of Wnt-mediated β-catenin signaling in MSC-mediated protection of CML progenitors from TKI.[195]

Despite the success of targeted therapies against BCR-ABL with the development of TKIs, a residual disease is still detected in many CML patients, probably due to the persistence *in-vivo* of CML LSCs. In some cases, resistance to TKIs treatment appears, involving different mechanisms with 30% of unknown origin that could be attributed to LSCs survival. LSCs are controlled by a variety of biochemical and biomechanical signals from the leukemic niche. Thus, deregulation of these signals could contribute to LSC emergence and resistance. In this context, co-culture systems have demonstrated that physical interactions (such as adhesion) between CML LSCs and their bone marrow stroma are involved in TKI resistance. Interestingly, cell adhesion is crucial for mechanical signals transduction. Moreover, recent studies also evidenced the key role of mechanical signals in the control of stem cell fate as well as in cancer processes. As such, mechanical signals provided by the bone marrow microenvironment could also be involved in the control of LSCs fate and resistance to TKIs treatment.

Chapter 3 : cell mechanics

I. Cell structure and stiffness

1. Introduction

Cell stiffness is given by their ability to resist to mechanical deformation. Cell intrinsic mechanical properties mainly rely on the organization and polymerization of filamentous proteins from the cytoskeleton. The cytoskeleton is composed of three distinct filament systems: microtubules, actin microfilaments, and intermediate filaments that are involved in the control of cell architecture, shape and stiffness. Microtubules and actin microfilaments are built from homogeneous globular proteins, called tubulin and actin, respectively, and serve as tracks for molecular motors. In contrast, intermediate filaments are made of diverse fibrous proteins. These three cytoskeletal filament systems are in constant link with each other through a group of proteins called cytolinkers.[196] Indeed, the disruption of intermediate filaments network organization can also disturb the spatial organization of microtubules or actin microfilaments.[197]

Interestingly, a recent study compared the contribution of each of these cytoskeletal components on chondrocytes mechanical properties.[198] Before mechanical testing, cytochalasin D, acrylamide, or colchicine were used to disrupt actin microfilaments, intermediate filaments, or microtubules, respectively. Cells were subjected to a range of compressive strains and allowed to recover to equilibrium. Inhibition of actin microfilaments had the greatest effect on bulk compressive stiffness while intermediate filaments and microtubules were each found to play an integral role in either the diminution (compressibility) or retention (incompressibility) of original cell volume during compression.[198]

2. Actin filaments

Actin cytoskeleton is a network of filaments of 6 nm in diameter that are important for anchoring plasma membrane proteins, for producing cell movement and for cell division. Inside cells, actin exists in two states, the monomeric protein, called G-actin for globular actin and the 6 nm filament, called F-actin for filamentous actin.[199] Thus, to understand the structure, dynamics and mechanical properties of actin networks in cells, one must consider the process by which F-actin is formed from G-actin and how it is regulated. Nucleation of F-actin polymerization requires the formation of an actin trimer from G-actin monomers bound with an energy-storing ATP nucleotide. This results in a nucleation rate that is slow due to the cubic dependence on actin concentration and also the required presence of ATP. The

cell can therefore regulate actin polymerization by varying the availability of actin, ATP or through the use of monomer-binding proteins that promote (profilin)[200] or inhibit (thymosin)[201] monomer binding to filaments or the ATP-ADP exchange. Actin filaments are in highest concentration near the surface of cells, in the actin cortex, where they often exist as short filaments of a few hundred nanometers, forming an isotropic cross-linked mesh. The F-actin cortex is largely responsible for controlling cell shape and dynamic surface motility and is also believed to play an important role in anchoring membrane proteins.[202] Longer microfilaments can also be organized into highly cross-linked bundles, called stress fibers, which serve as contractile elements within cells. In addition, these structures may be important for producing contractility to generate directional force during cell motility. Actin stress fibers can attain up to 10 μm and are part of the contractile apparatus in muscle cells and in non-muscle adherent cells. The formation of a third microfilament-based structure, the contractile ring, is critical for the separation of a cell into its two progenies during cytokinesis.

These cytoskeletal structures are important to maintain connections between the cells and their microenvironment, that is to say the surface on which they grow. In adherent cells, the extremities of actin filaments aggregate at specific locations on the plasma membrane, known as focal adhesions that link the cytoskeleton to the ECM via integrin receptors.[203–205] Thus, integrins located at the membrane form the link between ECM proteins such as fibronectin and intracellular actin cytoskeleton. On the intracellular side, many actin-binding proteins are involved in the integrin-actin connection such as talin, vinculin, α-actinin or filamin.[206] Other membrane-associated signaling molecules such as focal adhesion kinase, paxillin or src-kinase then regulate these proteins. Actin-membrane association is critical for mechanical integrity at the cell surface as well as for the coupling of external forces into the cell. Actin-binding proteins are capable to control F-actin cytoskeleton network, and consequently cell stiffness.[207] For example, bundling is achieved by parallel filament cross-linking by molecules such as fimbrin or α-actinin.[208,209] Meshes are formed by cross-linking at right angles (filamin) or branching (Arp2/3 complex, cortactin).[210] These differences in cytoskeletal structure can in turn translate into local effects on cell stiffness. Indeed, a recent study combining interference RNA (RNAi) to atomic force microcopy (AFM) in endothelial cells demonstrated that loss of cortactin or α-actinin-4 expression significantly decreased endothelial cell peripheral stiffness.[207] These results were also correlated by alterations of F-actin organization.

Moreover, F-actin usually associates with myosin, a molecular motor that converts chemical energy to mechanical energy though ATP hydrolysis, thus generating force and movement.[211] The myosin motor protein is considered the key effector in many cellular processes including cell division, muscle contraction or cell movement, thus playing a central

role in cell biology. The small myosin-I isoform can be anchored to a membrane or lipid vesicle and is therefore critical for trafficking of membrane proteins. The myosin-II isoform is larger and often self-assembles into thick filaments aligned parallel to long actin filaments, with many sites of interaction between the myosin heads and the actin filaments. These types of actomyosin interactions are termed cross-bridges, whose dynamic cycling is responsible for large force production and shortening of contractile fibers. It is well known from the literature that cell stiffness is determined in large part by the actin cytoskeleton.[212–215] Indeed, disruption of F-actin cytoskeleton with cytochalasin D or latrunculin B has been demonstrated to induce cell softening in different models.[216]

3. Microtubules

Microtubules are long, hollow cylindrical filaments, approximately 25 nm in diameter, and are responsible for large-scale cellular properties, such as organelle organization, stabilization of mechanical structures, force transmission across the cytoplasm, and processes like cell division.[217] They are the major constituents of mitotic spindles, which are involved in chromosome separation during mitosis and meiosis. Microtubules are formed from the polymerization of two globular proteins, alpha and beta tubulin. Their polymerization depends on the concentration of αβ-tubulin dimers and guanosine triphosphate (GTP) in solution. Tubulin dimers can bind two molecules of GTP, one of which can be hydrolyzed subsequent to assembly. During polymerization, the tubulin dimers are in the GTP-bound state. The GTP bound to α-tubulin is stable and it plays a structural function in this bound state. However, the GTP bound to β-tubulin may be hydrolyzed to guanosine diphosphate (GDP) shortly after assembly. The assembly properties of GDP-tubulin are different from those of GTP-tubulin, as GDP-tubulin is more prone to depolymerization. Microtubules are nucleated and organized by microtubule organizing centers (MTOCs), such as the centrosome found in the center of many animal cells. Contained within the MTOC is another type of tubulin, γ-tubulin, which combines with several other associated proteins to act as a template for α/β-tubulin dimers to begin polymerization.[217] Especially in axons and dendrites of neurons, microtubule associated proteins (MAPs) bind along the length of microtubules and stabilize them.[197] Important differences in the specific molecular interactions give rise to distinct kinetic and mechanical properties of microtubules. The motor proteins kinesin and dynein bind to microtubules and move in opposite directions. Because microtubules can span the entire length of a cell, kinesin and dynein are critically important in large-scale trafficking of proteins or vesicles along microtubules.[218]

Interestingly, differences in microtubules polymerization can affect local cell stiffness, as demonstrated in a recent study using carbon fiber-based systems to analyze the

mechanical properties of single cardiomyocytes.[219] The impact of microtubule organization was assessed by treating the cells with colchicine, a microtubule-depolymerizing agent, or with paclitaxel, a microtubule-polymerizing agent. It was found that stiffness depends on microtubule polymerization.[219,220] A simulation model of the myocyte in which microtubules serve as compression-resistant elements further confirmed the experimental results. Microtubules are involved in various pathological conditions of the heart including hypertrophy and congestive heart failure. Thus, these results suggests that changes in cardiomyocytes' mechanical properties may have a significant influence on the diseased heart that is subject to complex strain fields in the body.[219] Finally, taxol, a drug that facilitates microtubule polymerization, increased endothelial cell stiffness by 10%.[221]

4. Intermediate filaments

Contrarily to microtubules and actin microfilaments, intermediate filaments are apolar and are made of diverse fibrous proteins. Indeed, intermediate filaments share a common domain organization but have distinct primary sequences as over 70 human genes encode them. Intermediate filaments are divided into six major classes on the basis of similarities in sequence.[222] They are differentially expressed during development and exhibit cell and tissue specificity. For example, while epithelial cells express diverse keratins, mesenchymal, endothelial, and hematopoietic cells express vimentin, muscle cells express desmin and neuronal cells express neurofilaments.[222] Intermediate filaments are composed of polypeptide rods, arranged in rope-like fibers that are about 10 nm in diameter. They are known to resist rupture under large force. Indeed, a network of intermediate filaments is often found as a laminating layer adjacent to cellular membranes, where it provides mechanical support. They are concentrated around cell-cell junctions, but are also found in other parts of the cell including the nucleus.

The nuclear lamina is a structure located along the inner nuclear membrane and the peripheral chromatin. This supporting network is composed of lamin A and lamin C filaments cross-linked into an orthogonal lattice, which is attached via lamin B to the inner nuclear membrane through interactions with a lamin B receptor.[222] Lamins are involved in the regulation of most nuclear activities including DNA replication, RNA transcription, nuclear and chromatin organization, cell cycle regulation, cell development and differentiation and apoptosis.[223,224]

Specialized cell junctions called desmosomes and hemi-desmosomes hold epithelial cells of organs and skin together. Desmosomes mediate cell-cell adhesion,[217] and hemi-desmosomes are responsible for attaching cells to the underlying basement membrane.[225] The intermediate filaments in one cell are thus directly connected to the intermediate

filaments in a neighboring cell via desmosomes or to the ECM via hemi-desmosomes. As a result of the connections between intermediate filaments and these cell junctions, shearing forces are distributed from one region of a cell layer to the entire sheet of epithelial cells, providing strength and visco-elasticity to the entire epithelium. Intermediate filament-associated proteins (IFAPs) cross-link intermediate filaments with each another, forming a bundle called tonofilament or a network, and also with other cell structures including the plasma membrane.[226]

In epidermal cells, bundles of keratin, an intermediate filament, are cross-linked and anchored to desmosomes.[208] The structural integrity of keratin network has been demonstrated to be essential in order for the skin layer to withstand abrasion. Indeed, expression of mutant keratin proteins in cells causes intermediate filament networks to breakdown into aggregates.[227] Without their normal bundles of keratin filaments, mutant basal cells become fragile and easily damaged, causing the overlying epidermal layers to delaminate and blister. In mice, this leads to the appearance of gross skin abnormalities that resemble the human skin disease epidermolysis bullosa simplex.[227] Death of these cells appears to be caused by mechanical trauma from rubbing of the skin during movement of the limbs. Just like the role of desmin filaments in supporting muscle tissue, keratin filaments play a crucial role in maintaining the structural integrity of tissues by mechanically reinforcing the connections between cells.[228]

5. Membrane lipid composition

While cytoskeleton molecules have been identified as key elements in the regulation of cell structure and stiffness, other cellular component may also be involves in these processes. This is the case for example for lipid membranes. The lipid bilayer is a thin polar membrane made of two layers of lipid molecules with proteins embedded in it.[229] These membranes are flat sheets that form a continuous barrier surrounding all cells as well as their nucleus and other sub-cellular structures. Biological membranes typically include several types of lipids other than phospholipids. A particularly important example in animal cells is cholesterol, which helps strengthen the bilayer and decrease its permeability.[230] Indeed, a recent study investigated the effect of cellular cholesterol on membrane deformability of bovine aortic endothelial cells.[231] Cellular cholesterol content was depleted by exposing cells to methyl-beta-cyclodextrin or enriched by exposing cells to methyl-beta-cyclodextrin saturated with cholesterol. Mechanical properties of the cells with different cholesterol contents were then compared by micropipette aspiration. Cholesterol depletion of bovine aortic endothelial cells resulted in a significant decrease in membrane deformability indicating that cholesterol-depleted cells are stiffer compared to control cells.[231] Repleting the

cells with cholesterol reversed the effect. While cholesterol concentrations had no apparent effect on F-actin content, disrupting F-actin with latrunculin A abrogated the stiffening effect.[231] Thus, this suggests that cholesterol depletion increases the stiffness of the membrane by altering the properties of the submembrane F-actin and/or its attachment to the membrane.

6. Plasma membrane potential

Although the lipid membrane can control cell stiffness by interactions with underlying actin cortex, it is also involved in molecules trafficking inside and outside the cells. Indeed, ion pumps and ion channels embedded in the membrane regulate ions concentrations, thus controlling electric potential.[232] Plasma membrane potential is the difference in electric potential between the interior and the exterior of a cell. Interestingly, it was recently demonstrated that the electrical plasma membrane potential difference is involved in the control of endothelial cell stiffness.[233] Using a novel technique that combines fluorescence-based membrane potential recordings with atomic force microscopy based stiffness analysis, it was shown that membrane depolarization is associated with a decrease in the stiffness of endothelial cells. Moreover, experiments using actin-destabilizing agent cytochalasin D indicated that change of the electrical field across the plasma membrane acts by affecting cortical actin polymerization/depolymerization ratio and thus cell stiffness.[233] The stiffness of vascular endothelial cells is crucial to mechanically withstand blood flow and, at the same time, to control deformation-dependent nitric oxide release.[234,235] This depolarization-induced decrease in the stiffness of endothelial cells could play a role in flow-mediated nitric-oxide-dependent vasodilation, thus also impacting tissue stiffness.[236,237]

7. Impact of ECM on cell structure and stiffness

Cells are located in a microenvironment containing other cells, but also ECM elements. The ECM also contains key growth factors, such as angiogenic factors and chemokines that interact with cell surface receptors and give each tissue its tensile and compressive strength and elasticity.[238] Cellular interactions with ECM are mainly mediated by focal adhesions,[239] large and dynamic protein assemblies that connects ECM to the cell cytoskeleton, involved in the regulation of nearly every major cellular behavior, including growth, division, survival and movement.[240] Indeed, cells can sense not only the composition of the surrounding ECM, but also its topography, rigidity, and anisotropy.[241] Moreover, cells dynamically react to changes in applied force or tension generated by, or in response to ECM dynamics. Cells respond to extrinsic forces from the ECM by modifying their behavior, remodeling the ECM itself,[242] and exerting counter-tension through actomyosin-dependent

contractility.[203] In normal cells, this “mechano-reciprocity” is in controlled equilibrium and is important for tissue homeostasis. This concept implies that changes in microenvironment stiffness will affect cell stiffness and vice-versa, potentially affecting cell behavior.

II. Mechanical forces

Cells within tissues are continuously exposed to physical forces that participate in the control of their shape and fate. Depending on tissue type, physiological or pathological conditions, distinct forces will be exerted on cells by their environment (Figure 16). These forces are characterized by their magnitude, orientation, frequency and duration.[243–246]

Shear forces are induced by fluid flow over cell surfaces in blood vessels and cavities or by forces acting within tissues and act in parallel to the plasma membrane.[247] They are the main forces exerted in vascular system where the blood flow induces shear stress on endothelial cells, but are also prominent in bone marrow, as fluid flow exerts shear stress on stromal cells and adherent hematopoietic cells. Tensile forces can be generated by the internal tension of cell cytoskeleton, in link with ECM stretching or contraction of surrounding cell layers.[248] Compressive forces act contrariwise to tensile forces and can be generated by cell hypertrophy or cellular proliferation.[249] They are mainly observed in response to cell expansion during embryonic and tumor processes. Hydrostatic pressure results from gravity acting on fluid filled cavities and pores and from extrinsic forces acting inside and onto the surrounding tissue resulting from muscle contraction or body weight.[250] Similar to shear forces, hydrostatic pressure is very prominent in the vascular system. Finally, tissue stiffness will be determined by the tissue composition (cell types, ECM proteins), the degree of ECM cross-linking as well as the contractility of surrounding cells. Tissue stiffness is a mechanical signal that requires reciprocal force sensation by active cell body contraction. *In-vivo*, cells persist in a tensegrity-based force balance with their surroundings, which also determines their stiffness.[251] The nature of these forces can change in pathologies such as cardiovascular disease and cancer.

Because mechanical signals can influence cell structure and fate, this means that cells are able to sense mechanical signals and to translate them into biochemical signals involved in their regulation. As a result of research efforts from a variety of disciplines, we are beginning to understand the molecular basis of mechano-transduction process.

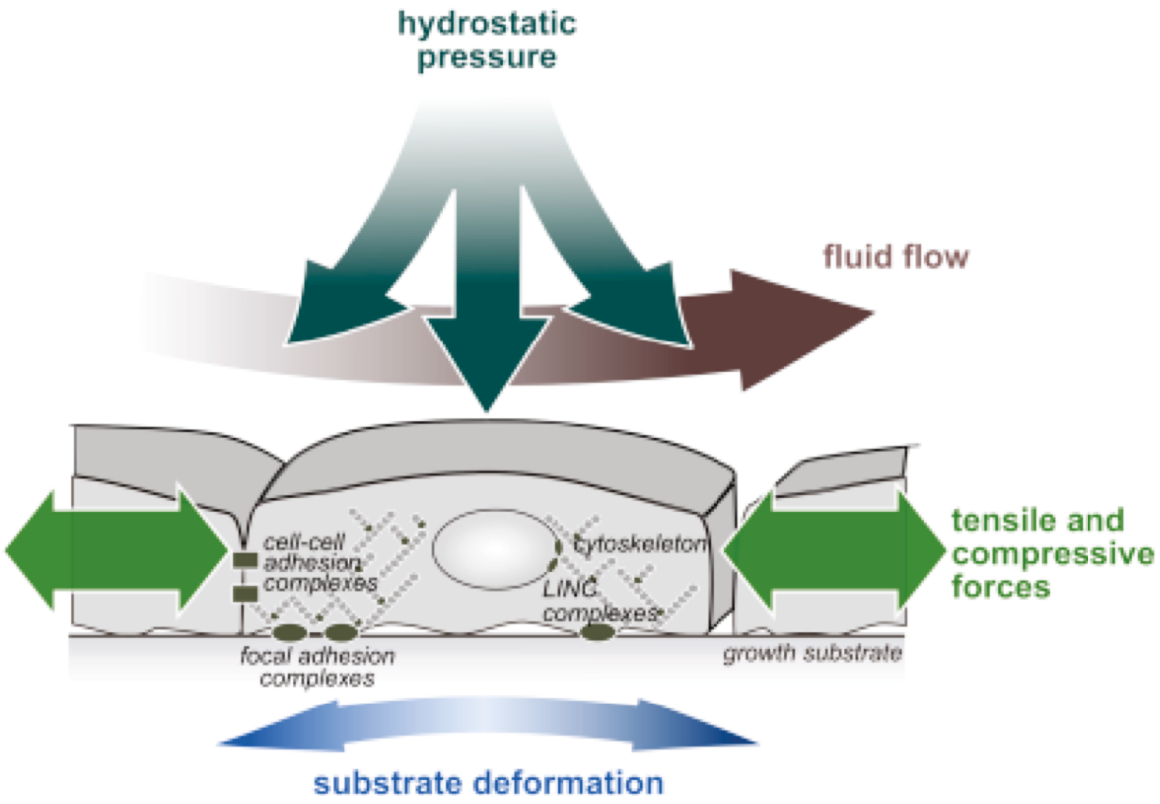

Figure 16 : Mechanical forces that act on the cellular level *in-vivo*. Collective cell-cell and cell-matrix interactions allow the propagation of mechanical forces within tissues. Extrinsic influences like fluid flow, hydrostatic pressure and substrate deformation lead to additional forces acting on the cells and result in cell straining. Tensile and compressive forces are generated by intrinsic cell-cell interactions in a tissue collective. Mechanical triggers are perceived on the cellular level by different subcellular structures, such as cell-cell adhesion, cell-matrix adhesion complexes, and different cytoskeletal and nucleoskeletal structures. Adapted from Kopf et al, 2013.

III. Mechanical signals transduction

The conversion of mechanical signals into biochemical cellular responses is termed mechano-transduction. While we are just beginning to understand how these mechanical signals are sensed and transduced into biochemical informations by cells, several mechano-transduction mechanisms have already been identified. The primary mechano-sensor initially responsible for the biochemical translation of mechanical forces also remains uncertain.

1. Integrin-mediated signaling

As the main receptors that connect the cytoskeleton to the ECM, integrins mediate cellular adhesion to the growth substrate and have been described as major cellular components to mediate force detection at the plasma membrane and to trigger pleiotropic mechano-transduction pathways.[252] Integrins convey mechanical forces from ECM to the cytoskeleton, thus controlling many different signaling pathways. Thus, it has been

suggested that integrin-dependent molecular signaling is a key signaling platform for cellular responses to various forms of mechanical stress, including cell adhesion.[252] Indeed, many of the signals triggered by physical forces are consistent with known effects on focal adhesions such as increased integrin clustering and focal adhesion kinase (FAK) phosphorylation, that can in turn control several downstream pathways like ERK or JNK signaling.[205]

2. Stretch Activated Channels (SACs)

Mechano-sensitive ion channels that become more permeable to soluble ions in response to mechanical signals are supposed to function through a protein 'gate' which is physically separated under force.[253] For example, TRP calcium ion channels were identified for their ability to permit transient calcium influx in stretched cells.[254] It has been shown that cells cultured on substrates with different stiffness exhibit changes in the amplitude of calcium ion oscillations, demonstrating that SAC permeability is related to active sensing of the cell physical microenvironment.[232] Furthermore, cells plated on substrates with a stiffness gradient showed even stronger calcium ion oscillations. While changes in calcium ion concentration can affect a number of signaling pathways, other mechano-sensitive channels, such as the TRAAK potassium ion channels, are also activated upon mechanical stress-induced membrane deformations.[255]

3. Force-induced protein unfolding

Force-inducible proteins are capable of unfolding under applied force, exposing binding domains for other proteins and signaling molecules. For example, talin, a focal adhesion protein that binds to integrins and actin, has been shown to unfold under stretching forces as low as 12 pN and expose up to 11 binding domains for vinculin, another focal adhesion protein.[256] Furthermore, the focal adhesion protein p130Cas, which binds to FAK and associates with talin, has been shown to unfold under applied force to activate the Crk/C3G-Rap1 signaling cascade resulting in activation of MAPK pathway.[257] Another protein, receptor-like tyrosine phosphatase alpha (RPTPα) has been shown to associate with α integrins, catalyzing binding to fibronectin and vitronectin in a force-dependent manner via the Src family kinase cascade.[204] Thus, a connection can be made between force-dependent unfolding of focal adhesion proteins and signaling cascades that may ultimately affect gene expression.

4. Control of ligand bioavailability

Cell regulation does not only occur via secreted molecules, but also through ligand presentation by the ECM. For example, both TGF-β and BMP ligands can bind to ECM

proteins.[258–261] Depending on the ECM composition, the degree of crosslinking as well as the deformation in response to force, bioactivity and bioavailability of soluble ligands may be altered. Such mechanism has been already identified for TGF-β ligands.[262–264] Following secretion, TGF-β binds to latent TGF-β binding proteins (LTBPs) via its pro-peptide.[262] Then, LTBPs anchor TGF-β to the ECM leading to the repression of TGF-β bioactivity.[262] However, in response to mechanical signals, integrin-dependent unfolding of this complex leads to a release of active TGF-β ligands.[265,266] A related mechanism, involving ECM molecules in the regulation of ligand bioavailability, might also apply for BMP ligands, but experimental studies have yet to be been performed. Furthermore, both TGF-β and BMPs themselves regulate SC fate[267] and thereby the composition and mechanical properties of the ECM.[268,269] Consequently, this represents a reciprocal interplay between BMP signaling, ECM production and mechano-transduction.

5. Mechano-sensitive genes control

Finally, mechano-sensing can be directly coupled to the nuclear envelope and thus to transcriptional regulation. The cytoskeleton is anchored to the nucleus via linker of nucleoskeleton and cytoskeleton (LINC) complexes, which contain nesprins, sun, and lamin proteins.[270] LINC complexes form an integral part of the nuclear envelope and are connected to networks and filaments in the nucleus. Although mechanistically still unclear, force transmission through cytoskeletal structures in the cytosol and via LINC complexes regulate chromatin dynamics and may therefore act as mechano-transducer upon cellular deformation.[271] In addition, mechanical stretch has been shown to regulate the expression and localization of chromatin remodeling enzymes called tension-induced proteins (TIPs).

For example, it has been demonstrated that mechanical stress is sufficient to trigger TIP1 expression and nuclear localization in embryonic mesenchymal SCs, thus regulating the expression of lineage-specific genes.[272] While many different genes have been described to be controlled by mechanical signals, most of these genes identified so far appear to be linked with stemness properties, such as *c-fos*,[273] *c-jun*,[273] *Twist-1*[274] or BMP-related genes.[243]

IV. Stem cell fate control

To date, many genes associated with SC properties have been found to be controlled by mechanical signals. However, the role of intracellular tension[275] and extracellular biophysical factors[276] has only recently been acknowledged as critical determinant of cell biology and SC fate.[277]

Interestingly, it has been demonstrated that cell substrate stiffness is able to control

mesenchymal SC differentiation toward specific lineages.[278] Indeed, when these cells were cultured on moderately stiff matrices (11 kPa, muscle-like stiffness), they engaged toward myogenic lineage, while culture on stiffer matrices (34 kPa, bone-like stiffness) led to their engagement toward osteoblastic lineage (Figure 17). Thus, these results indicate that SC engagement into a specific lineage depends on the presence of a matrix reproducing the original tissue's stiffness. Finally, as inhibition of myosin II led to the inhibition of all elasticity-directed lineage specification, this suggests a crucial role for actin-myosin cytoskeleton in the control of SC fate through transduction of mechanical signals.[275,278] Since then, numerous studies have confirmed the role of mechanical signals in SC differentiation.

In addition, mechanical stress has also been demonstrated to induce human embryonic SC self-renewal while inhibiting their differentiation.[279] It was further demonstrated that mechanical signals must act synergistically with biochemical signals to influence SC fate. Interestingly, culture of human embryonic SC on soft substrates also led to an increase of their self-renewal potential by maintaining high levels of Oct3/4, Nanog and Alkaline Phosphatase (AP) activities.

In another study, polyacrylamide gels with different stiffness were used to culture human mesenchymal SC. Using this strategy, it was demonstrated that mesenchymal SC remained quiescent when cultured on soft substrates but retained their proliferation and differentiation capabilities.[280]

Finally, mechanical signals can also promote SC proliferation. This was particularly well illustrated in an *in-vivo* study were low magnitude mechanical signals (LMMS) were applied on C57BL/6J mice with a vertically oscillating platform.[281] In this study, six weeks of LMMS increased the overall BM stem cell population by 37% and the number of mesenchymal SCs by 46%. Concomitant with the increase in SC number, the differentiation potential of mesenchymal SCs was biased toward osteoblastic and against adipogenic differentiation, as reflected by up-regulation of the transcription factor Runx2 and down-regulation of PPARγ.[281]

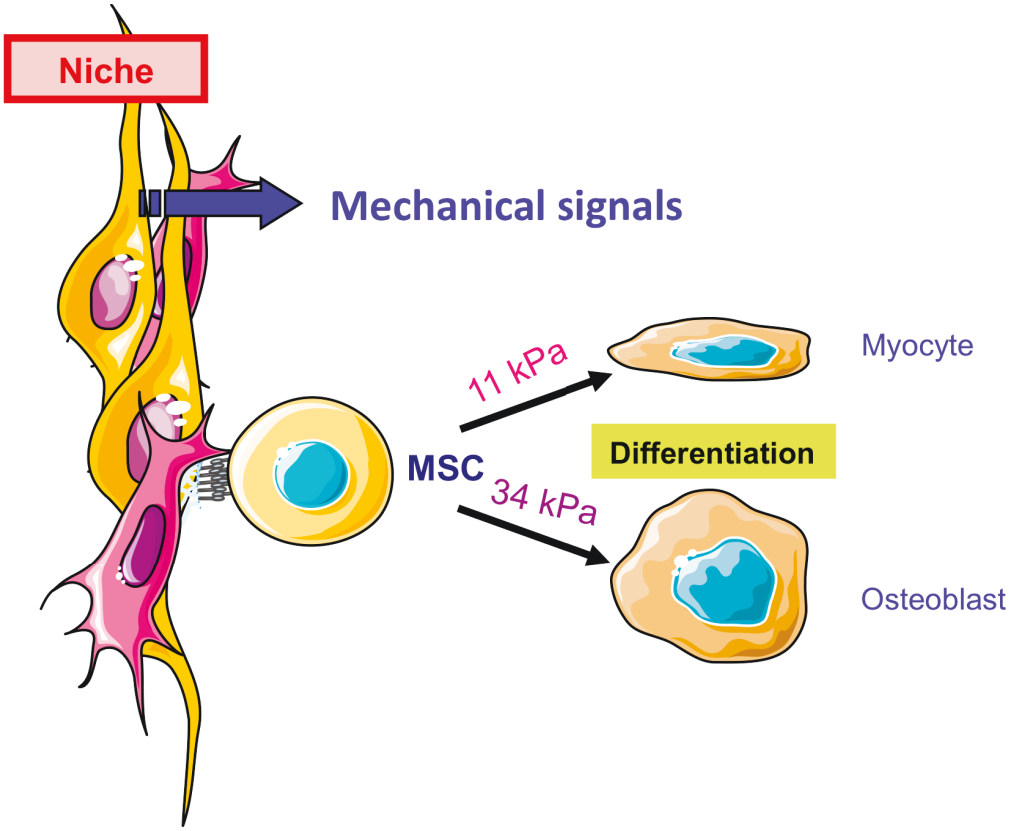

Figure 17 : Mechanical signals control stem cell fate. The control of stem cell fate by mechanical signals has been particularly well demonstrated in mesenchymal stem cells (MSC), thanks to the research performed to understand arthritis appearance, phenomenon thought to be linked to mechanical signals. Mesenchymal stem cells were cultured on substrates with different stiffness, leading to engagement into different cell lineages.

Although the crucial role for cell mechanics in SC control has been observed, the potential role of mechanical signals in the control of CSC fate has yet to be determined. Moreover, recent studies evidenced alterations of cancer cell intrinsic mechanical properties, which could potentially be associated with alterations of cancer cell response to external mechanical signals.

V. Cell stiffness and cancer

1. Probing cell mechanical properties

With the recent advances in biomechanics and nanotechnology, it has now become possible to probe single living cell stiffness. For example, biophysical tools such as atomic force microscopy,[282] micropipette aspiration or optical stretcher have been used to probe cell mechanics.[283] Young's modulus or elastic modulus (E) has been extensively used to measure cell mechanical properties.[284] Young's modulus describes tensile elasticity, that is the tendency of an object to deform along an axis when opposing forces are applied along

that axis. It is defined as the ratio of tensile stress to tensile strain (E = tensile stress / tensile strain). A material is said to be elastic if it deforms under stress and returns to its original shape when the stress is removed. The relationship between stress and strain (force and deformation) is linear and the deformation energy is returned completely. A stiffer material will display a higher elastic modulus. However, cell response to external force may also contain a viscous component. Viscoelasticity is the property of materials that exhibit both viscous and elastic characteristics when undergoing deformation. Viscosity is a measure of the resistance of a fluid to being deformed by either shear stress or extensional stress. The relationship between stress and strain is non-linear for viscoelastic material and the deformation energy is not returned completely. Shear modulus or modulus of rigidity (G) is the elastic modulus we use for the deformation which takes place when a force is applied parallel to one face of the object while the opposite face is held fixed by another equal force. It is defined as the ratio of shear stress to shear strain (G = shear stress / shear strain). The shear modulus is part of the derivation of viscosity and is also used to measure cell mechanical properties.[285] The bigger the shear modulus the more rigid is the material since for the same change in horizontal distance (strain) you will need a bigger force (stress).

2. Alteration of cancer cell stiffness

Tumor process is usually characterized by a palpable stiffening of the tissue. However, little is known about the effects of cell transformation and compaction on single cell stiffness. Over the past decade, there has been growing evidence indicating that the mechanical properties of cancer cells are altered. Many studies suggests that the increased deformability of cancer cells, due to an altered F-actin cytoskeleton could be involved in their migration and invasion capabilities.

In 2007, a comparative stiffness analysis of live metastatic cancer cells obtained from the body pleural fluids was performed in patients with lung, breast and pancreas cancer by atomic force microscopy (AFM).[286] Within the same samples, it was found that metastatic cancer cells were more than 70% softer compared to benign cells that line the body cavity. This work shows that mechanical analysis can distinguish cancerous cells from normal ones even when they display similar shapes. These results indicate that nanomechanical analysis correlates well with immunohistochemical testing currently used for the detection of cancer. Consistent with this study, the same group then demonstrated that metastatic tumor cells are more than 80% softer than benign cells using a cytocentrifugation method that yields morphologically indistinguishable cells.[287] Thus, this suggests that it is unlikely that morphology alone is sufficient to explain the differences observed between healthy and metastatic cancer cells. In addition, another study also demonstrated that metastatic ovarian cancer cells are softer than non-metastatic ones.[288] Moreover, it has been observed that PC3

prostate cancer cells are softer compared to non-tumorigenic PNT2 prostate cells.[289] In another AFM study, the mechanical properties of two mammary epithelial cell lines were compared. It was demonstrated that tumoral MCF-7 cells are softer compared to benign MCF-10A cells.[283] These cell stiffness alterations were correlated with differences of F-actin polymerization, and were also confirmed in another study using a microfluidic optical stretcher.[290] However, these results cannot be generalized for breast cancer cell stiffness as no other cell lines were tested. Moreover, these two cell lines are not very informative as MCF-10A cells were obtained from a patient with fibrocystic disease and MCF-7 were derived from a metastatic pleural effusion site. Nevertheless, these results were confirmed in other studies comparing the deformability of MCF-10A, MCF-7 and MDA-MB-231 breast cancer cell lines by optical stretching, further demonstrating that cell stiffness decreased with acquisition of metastatic competence.[291,292] Finally, a magnetic tweezer study demonstrated that ovarian cancer cells with the highest migratory and invasive potential are five times softer than cells with lower invasion potential.[293] It was further shown that decreasing cell stiffness by pharmacologic inhibition of myosin II increased invasiveness, whereas increasing cell stiffness by restoring expression of the metastasis suppressor TβRIII/betaglycan decreased invasiveness. These findings are the first demonstration of the relation between the stiffness and the invasiveness of cancer cells and show that mechanical phenotypes can be used to grade the metastatic potential of tumor cell populations.[293]

While the aforementioned studies demonstrated that cancer cells are softer than healthy ones, they restricted their analysis to metastatic cells, often highly structurally rearranged. Thus, we cannot conclude for generalized decreased cell stiffness in cancer, except for advanced metastatic processes. However, one of the first studies analyzing the mechanical properties of tumor cells demonstrated a decreased stiffness in cancer bladder cells compared to normal counterparts by scanning force microscopy.[294] Another interesting study recently demonstrated that transfection of healthy rat intestinal epithelial IEC-18 cells with c-K-ras oncogene was sufficient to decrease their stiffness.[295] An increase in cell stiffness heterogeneity was also observed.

Moreover, opposite differences were also observed. Indeed, in a recent study, a microfluidic device was used to electrically measure volume and transit time of single lymphocytes obtained from healthy donors and CLL patients.[296] By testing thousands of cells, it has been demonstrated that CLL lymphocytes are stiffer as compared to healthy ones, which was also confirmed by AFM indentation analysis. This observation is in sharp contrast with precedent studies performed on adherent metastatic cells. However, the underlying molecular mechanism involved in alteration of cell stiffness has not been identified. While difference in F-actin density was often thought to be responsible for deformability differences, no connection was found between actin level and cell deformability in human CLL cells.

Since cell nuclei are known to be significantly stiffer compared to cytoplasm and cell membrane,[297] the enlarged nuclei observed in CLL cells could contribute to the longer transit time observed in microfluidic channels.[296] One of the vascular complications observed in acute leukemia is leukostasis that occurs when leukemic cells accumulate in the microvasculature and damage vital organs. Previous work has shown that leukemic blasts are stiffer than their more mature counterparts and have suggested that cell deformability may play a significant role in leukostasis.[298] Interestingly, a recent study demonstrated that increased leukemic cell stiffness is associated with symptoms of leukostasis in pediatric ALL.[299]

Furthermore, another study also reported the impact of chemotherapy on leukemic cell mechanical properties. After exposure to dexamethasone or daunorubicin, ALL and AML cells' stiffness was analysed using AFM or microfluidic channels.[300] The results demonstrated that chemotherapy exposure increases leukemic cell stiffness. However, even if this stiffness increase was observed before cell death, apoptosis has been involved in alterations of cell stiffness but induced opposite results depending on the cell type studied.[300,301] Thus, it would be interesting to check if cells that do not die upon chemotherapy also display an increased stiffness in response to chemotherapy, to ensure that this is not an artefact related to cell death. As deformability of blood cells is known to influence vascular flow and contribute to vascular complications, these observations suggest that chemotherapy itself may increase the risk of vascular complications in acute leukemia.[300] However, the opposite effect was observed after treatment of B-lymphoma cells with monoclonal antibody rituximab.[302] In that case, the cells became dramatically softer after treatment. While the underlying mechanism has not been identified, the authors suggested a potential effect for CD20 in the remodeling of cell membrane and cytoskeleton after binding of rituximab.[302]

Interestingly, differences in cell mechanical properties have been proposed as prognostic markers not only for metastatic potential but also for response to anti-tumoral treatment. Indeed, a recent study used AFM to compare the mechanical properties of circulating tumor cells from prostate cancer.[303] It was demonstrated that castration-resistant prostate cancer cells were three times softer compared to castration-sensitive cells. This suggests that mechanical properties of metastatic cells may serve as novel and effective biomarkers to detect the presence of castration resistant prostate cancer. However, no clues were provided on the molecular basis of the differences observed. The potential of cell mechanics in prognosis was confirmed in ovarian cancer cells where sensitive cells showed dose-dependent increase in cell stiffness upon cisplatin treatment while no differences were observed for resistant cells.[304] This effect was correlated with alterations of F-actin cytoskeleton.

Although the contribution of the cell mechanical properties in invasion processes is not completely clear, it was recently evidenced that greater elasticity of cancer cells helps them metastasize by squeezing through the body tissues and capillaries.[305] Up to now, while alterations of cell mechanical properties were observed in different types of tumors, cancer cells were often found to be softer compared to healthy ones (Figure 18). Interestingly and at the opposite of solid tumors, leukemic cells tend to become stiffer compared to healthy ones, as observed in CLL and ALL processes. However, this needs to be confirmed in other hematologic malignancies. Moreover, the structural alterations responsible of cell stiffness alterations are often unknown, even if alterations of F-actin have often been observed.[305]

Cancer type	Cell specificities	Technique	Impact on cell stiffness	Structural alterations
Breast	metastatic cell lines	microfluidic channels optical stretcher AFM	-	F-actin microtubules stability
	primary metastatic cells	AFM	-	/
Lung	primary metastatic cells	AFM	-	/
Pancreas	primary metastatic cells	AFM	-	/
Ovarian	metastatic cell lines primary cells	AFM magnetic tweezer	-	TGF-β receptor-mediated F-actin alteration
	cisplatin treated cells sensitive cells only	AFM	+	F-actin
Prostate	metastatic cell line	AFM	-	/
	primary castration-resistant circulating tumor cells	AFM	-	/
Colorectal	oncogene transduction in rat cell line	AFM	-	c-K-ras oncogene
Bladder	cell lines	AFM	-	v-ras oncogene
CLL	primary cells	AFM microfluidic channels	+	enlarged cell nucleus
AML	Chemotherapy treated primary cells and cell lines	AFM microfluidic channels	+	F-actin
ALL				
pediatric ALL	primary cells from patients with leukostasis	AFM	+	/
B-lymphoma	Rituximab treated primary cells	AFM	-	CD20-mediated cytoskeleton alteration

Figure 18 : Alteration of cell mechanical properties in cancer. Table summarizing the alterations of cancer cells mechanical properties identified so far. The cells' specificities, the techniques used, the impact on cell stiffness and the possible identified structural alterations are listed. A "minus" represents a decrease of cell stiffness in cancer cells, a "plus" an increase.

Because of mechano-reciprocity between cells and their microenvironment, alteration of cancer cell stiffness could also impair microenvironment stiffness as well as the mechanical forces perceived by adherent neighbor cells (through cell-ECM or cell-cell interactions). In tumors, mechanical changes in the environment (compressive stress due to tumor growth or ECM alterations) are supposedly exploited by tumor cells to facilitate local

growth, invasion, and spread.[241] Thus, cancer cell stiffness modifications could lead to an alteration of mechanical signals production and to an alteration of cancer cells response to mechanical signals.

VI. Alteration of mechanical signaling in cancer

Despite the crucial role of mechanical stress in physiological processes such as cell growth, differentiation, and apoptosis, it can also be involved in pathological processes. Alteration of external mechanical signals in tumors is often detected as a palpable 'stiffening' of the tissue. This observation appears in contrast with the decreased stiffness previously described in cultured cancer cells and biopsies from cancer patients.[287] This paradox is associated with high remodeling and stiffening of the peripheral tumor stroma resulting from the accumulation of aberrant ECM deposition and organization.[306–312] For example, breast tumorigenesis is accompanied by collagen cross-linking leading to ECM stiffening.[308] This stiffening may be partially attributed to cancer-associated fibroblast (CAFs)[313] secreting lysyl oxidase (LOX), an enzyme that cross-links collagen IV found in ECM. ECM stiffening forced integrin clustering, promoted focal adhesions, enhanced PI3K signaling, and increased tumor invasive properties.[314]

Interestingly, a recent study demonstrated that the expression of both Myc and Twist1 oncogenes is increased in response to transient compression in APC deficient colon tissue explants, but not in wild-type colon explants.[315] The mechanical activation of Myc and Twist1 expression in APC deficient colon can be prevented by blocking β-catenin phosphorylation using Src kinase inhibitors. Microenvironmental signals are known to cooperate with genetic lesions to promote the nuclear β-catenin accumulation that drives colon cancer. These results suggest that mechanical stimulation produced during intestinal transit or tumor growth could be interpreted by cells of preneoplastic colon tissue as a signal to initiate a β-catenin dependent transcriptional program leading to cancer initiation.[315]

In cancer processes, and particularly in solid tumors, compressive mechanical stress is produced during cell growth in a restricted matrix.[249,316,317] This was demonstrated in a study where single cancer cells were co-embedded with fluorescent micro-beads in agarose gels. During spheroid growth, changes in micro-bead density allowed to detect an increase of mechanical stress during tumor growth. This was further correlated with a suppression of cell proliferation and induction of apoptotic cell death through the mitochondrial pathway in regions of high mechanical stress.[317] Thus, mechanical stress increases in tumor context, and probably affects tumors growth by controlling cell functions. Interestingly, inhibition of spheroids growth under mechanical stress had already been observed, but was accompanied by a decrease of both cell proliferation and apoptosis.[318,319] However, opposite

effects have been suggested in mice studies. High tumor interstitial fluid pressure (TIFP) is a characteristic of most solid tumors that generates mechanical forces affecting the tumor cortex.[249] Solid epithelial tumors were grown in nude mice generating different ranges of TIFP. Strikingly, tumor drainage led to a rapid decline of TIFP, together with visible relaxation of the tumor cortex and a decrease of tumor cell proliferation. In addition, molecular analysis showed a decreased phosphorylation of proliferation-associated p44/42 mitogen-activated protein kinase and of Ki-67 proliferation marker after TIFP lowering.[249] These data suggest that mechanical stress induced by TIFP is a positive modulator of tumor proliferation. These results are in line with another study demonstrating that extracellular pressure stimulates colon cancer cells proliferation by activating protein kinase C alpha (PKCα) signaling.[320,321]

Finally, another study demonstrated that compressive stress stimulates migration of mammary carcinoma cells.[322] The enhanced migration under compression was accomplished by a subset of cells displaying localized fibronectin deposition, stronger cell-matrix adhesion and stabilization of persistent actomyosin-independent cell extensions. This suggests that compressive stress accumulated during tumor growth can induce coordinated migration of cancer cells by stimulating enhancing cell-substrate adhesion and migration.

In conclusion, while the role of cell mechanics just begins to be understood, mechanical stress has been reported to play a role at al levels of tumorigenesis, including tumor initiation,[315] control of tumor size[318,319] and tumor progression.[322] So far, little is known about how mechanical stress affects cancer cell phenotype or the molecular pathways involved. However, BMP signaling has been described as a key pathway in the transduction of compressive mechanical signals produced during tumor growth.

Chapter 4 : BMP signaling pathway

I. Discovery

The bone morphogenetic proteins (BMP) were discovered in the 1960s from extracts of demineralized bone marrow.[323] These molecules have been identified by their osteoinduction ability, that is to say, their capability to induce the formation of bone structures and are now widely used in orthopedic surgery.[324,325] BMP are glycosylated extracellular matrix-associated molecules that belong to the transforming growth factor beta (TGF-β) superfamily.[326] The molecules of the TGF-β superfamily are usually classified according to their sequence homology. Because of this, BMPs are often associated with growth and differentiation factor (GDF) molecules.[327] BMPs have highly conserved molecular structures in animals. Recently, scientific advances have led to the isolation and cloning of these molecules allowing to refine their classification. BMPs can thus be grouped into sub-classes, as it is the case for BMP2 and BMP4 that share more than 80% of sequence homology.[328] BMPs are generally described as "multifunctional" proteins. They can regulate many cellular processes (proliferation, differentiation, apoptosis, chemotaxis...) in a variety of different cell types (hematopoietic, epithelial, mesenchymal, neural...) both during embryonic development and in adult tissues.[329] BMPs have been widely studied in embryonic development and in bone remodeling related processes and therefore it is logical that their biological roles are best known in these two areas. It is now clear that this unique and essential signaling pathway plays a major role in physiological as well as in pathological processes.[330]

II. BMP signaling pathway

BMPs ligands are secreted as larger precursor molecules with an N-terminal latency/signal peptide,[331] which is cleaved by extracellular proteases such as furin to release the mature protein.[332] The mature protein dimerizes to form the bioactive complex. BMP molecules can form homo- or hetero-complexes that will allow signal transduction within the cells.[333]

Main track BMP signaling, called canonical pathway, involves type II receptors with serine/threonine kinase activity (BMPRII) on which binds the ligand and type I receptors (BMPRI) determining the specificity of the dimer. Three different type II receptors (BMP receptor II [BMPRII], activin receptor II [ACTRIIA and ActRIIB]) and three type I receptors (also called activin-like receptor kinase [ALK]: BMPRIa [ALK3], BMPRIb [ALK6] and ACTRIa [ALK2]) have been identified.[334] Type II receptors possess similar structures. However,

among type I receptors, ALK2 has a different structure from that of BMPRIa and BMPRIb. Indeed, it has been demonstrated that the extracellular region structure of BMPRIa and BMPRIb gives them greater affinity for both BMP2 and BMP4. Upon ligand binding, a hexameric complex of two type I receptors, two type II receptors, and a BMP ligand dimmer is formed, resulting in the phosphorylation and activation of type I receptors. BMPRI phosphorylation by BMPRII triggers the cascade phosphorylation of Smad effectors[335] (small mothers against decapentaplegic homolog) 1, 5 and 8, the formation of a complex with Smad4 that relocates into the nucleus where it binds to co-factors and transcription factors[336,337] to regulate the transcription of target genes such as *Id* (Inhibitor of differentiation)[338] and *Runx* (Runt-related)[339] genes (Figure 19). This complex either directly bind to Smad-binding elements (SBE), or indirectly through interactions with DNA-binding transcription factors and by associating with co-activators/co-repressors and histone-modifying factors.

Recently, different signaling pathways independent of Smad proteins, named non-canonical pathways, have been implicated in BMP signaling and can for example involve ERKs (extracellular-signal-regulated kinases), JNK (c-Jun N-terminal kinase), MAPK/p38 (mitogen-activated protein kinase), phosphoinositide (PI)3 kinase/Akt, protein kinase C (PKC), and Rho-GTPases signaling pathways (Figure 19).[340–345]

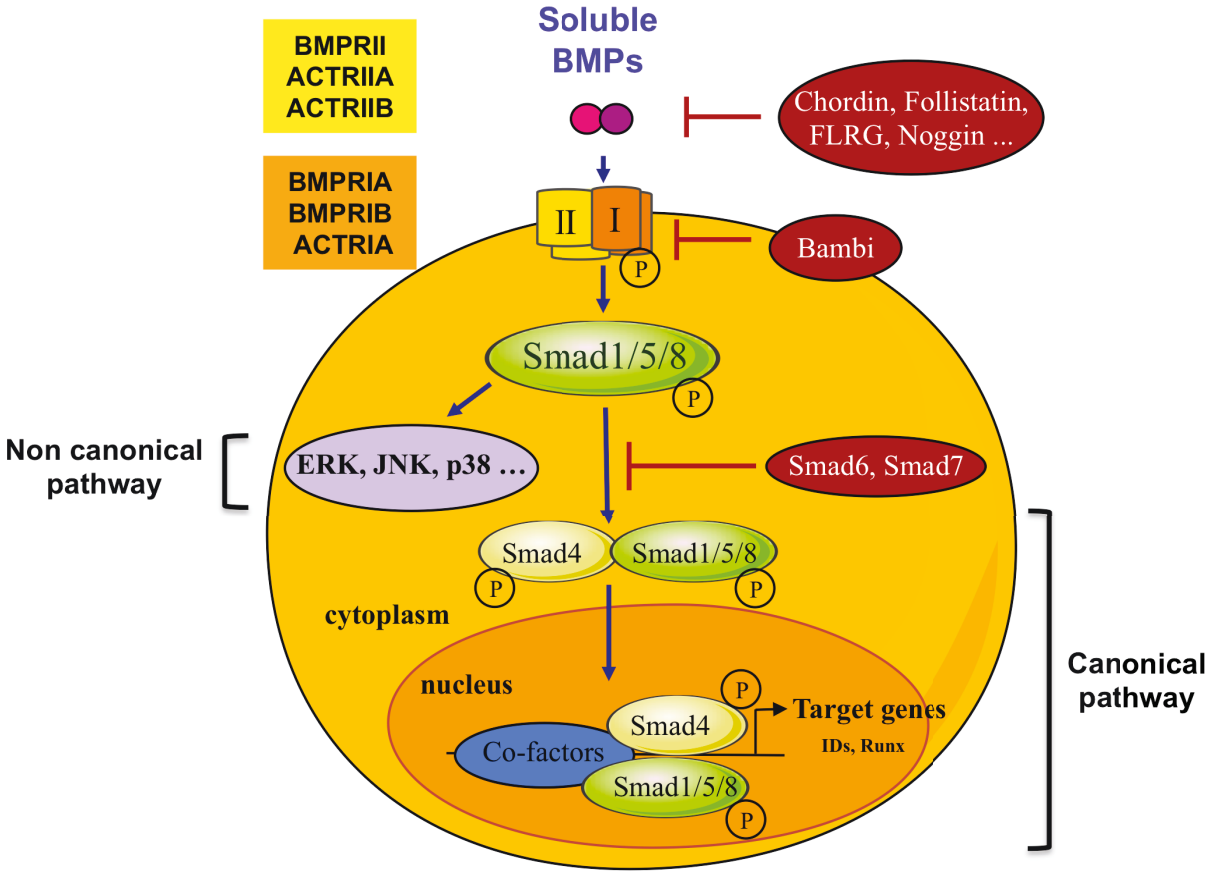

Figure 19 : BMP signaling pathway. Representative scheme of the BMP signaling pathway that displays the main components of the signaling cascade including extracellular (soluble molecules), membrane, cytoplasm and nuclear located responding elements as well as some BMP target genes. Canonical and non-canonical pathways are shown in this figure.

These signaling pathways are tightly controlled by a set of soluble, membranous and cytoplasmic regulators. The BMP pathway is inhibited by inhibitory Smads (Smad6 and Smad7),[346] which inhibit the phosphorylation of Smad effectors and recruit Smad ubiquitin ligases (Smurf1 and Smurf2) to promote the proteasomal degradation of receptors and Smad effectors.[347,348] Smad7 inhibits all TGF-β family members,[349] while Smad6 is more selective towards BMP family members.[350] BMP signaling is also controlled by secreted BMP antagonists able to bind to BMPs and prevent their engagement with their cognate receptors, such as chordin, follistatin, FLRG or noggin.[351,352] In addition, co-receptors such as endoglin, betaglycan or RGM family members can modulate the interactions between BMP ligands and receptors.[353,354] BMPs can also bind to the decoy receptor BMP and activin membrane-bound inhibitor (BAMBI), that resembles the type I receptors but lacks an active kinase domain and consequently sequesters ligands from the active receptors and inhibits BMP signaling.[355] The co-factors that will be recruited in the nucleus may influence the regulation of target genes, thus leading to different functional effects such as cell proliferation,

differentiation or even self-renewal.[336,337] Moreover, it has been demonstrated that phosphatases such as PP1 and PP2A are able to attenuate BMP signaling by dephosphorylation of both receptors and Smad effectors.[356] Recent studies have also demonstrated that BMP pathway is regulated by epigenetic mechanisms of hypo- and hyper-methylation of genes promoters, as well as through microRNAs.[357–359]

Furthermore, crosstalk exists between and BMP and other signaling pathways such as Wnt, JAK/STAT, Ca^{2+}/Calmodulin or TGF-β.[360,361] For example, although conventionally thought to respond to BMP signals, it has been demonstrated that Smad5 is also capable to transduce the inhibitory signal of TGF-β1 and TGF-β2 on proliferation of human hematopoietic progenitor cells.[362] Finally, a growing number of adapter proteins and interactions with other signaling pathways have been evidenced,[363] which demonstrates the importance and complexity of the BMP pathway.

III. Mechanical signals transduction through BMP signaling pathway control

Mechanical signals are integrated into the BMP signaling pathway by modulating BMP pathway elements expression or by direct modulation of BMP signal transducers. Bioreactor systems allowing cyclic compressive loading have been developed to study bone cell compliance in three-dimensional environments. Using this strategy, it has been demonstrated that early BMP signaling events are strongly potentiated by mechanical forces in human fetal osteoblasts.[364] Indeed, Smad1/5/8 phosphorylation increased both in intensity and duration under BMP2 stimulation with concurrent mechanical loading. In addition, Smad-independent pathways involving MAPK and Akt/PKB were initiated, as shown by p38, Erk1/2 and Akt phosphorylation. Conversely, in some cases, mechanical signals can also suppress BMP-mediated signaling. For example, it has been demonstrated that cell stretch using a Flexcell system suppresses the BMP4 induction of mesenchymal SC adipogenesis potentially through ERK up-regulation but not via the down-regulation of Smad or p38/MAPK signals.[365]

Mechanical signals can directly influence BMP ligands expression leading to an alteration of autocrine signaling. It has been shown that mechanical loading leads to a transcriptional regulation of several BMP ligands, such as *BMP2, BMP4, BMP6* and *BMP7*.[243,364,366,367,367,368,368–371] However, these molecules were found up-regulated in some studies, while their expression was decreased in other studies in response to mechanical stress. These differences probably depend on cell context, experimental setup as well as duration and quality of mechanical signals provided. Expression analysis revealed that mechanical loading also controls TGF-β superfamily antagonists such as *noggin, sclerostin, gremlin* and *follistatin*.[367,372–374] Moreover, while this has not been very well documented yet,

mechanical signals can impact the expression of intrinsic BMP signaling pathway elements as it was evidenced for *Smad6* gene.[375]

Interestingly, mechanical signals can also control endocytosis of membrane receptors, a process involved in the regulation of ligand-receptor interaction and degradation. Thus, receptor internalization is a common means to regulate signaling intensity and duration. For example, it has been evidenced that hypoosmotic-induced mechanical stress inhibited BMP2 endocytosis thus leading to an increase of Smad1 nuclear translocation in mouse pluripotent mesenchymal cells.[376] Interestingly, endocytosis is also connected to mechano-biological components. The potential role for BMP receptors in mechanical signal transduction has been proposed because of their co-localization with integrins,[377,378] that are key transducers of mechanical signals. Indeed, it has been demonstrated that endocytosis of integrin receptors depends on ECM stiffness and that altered endocytosis can also affect BMP receptor endocytosis and signaling.[379] Recently, it has been shown that endocytosis of integrin receptors depends on extracellular matrix stiffness and that this altered endocytosis also affects BMP receptor endocytosis and signaling.[379]

Finally, oscillatory shear stress was demonstrated to sustain BMPRIb and α5β3 integrin interaction, leading to an increase of Smad1/5 activation through the Shc/FAK/ERK pathway.[378]

IV. Functions

1. Biological functions

To date, over 20 different BMP molecules have been identified,[380] that are involved in an impressive number of biological processes in many tissues during embryonic development but also in adults tissues. However, given the functional overlap and redundancy between the soluble ligands and the lack of knowledge about the specification of soluble BMP through one or another receptor, the biological function of this pathway has proved very difficult to decode. In a normal context, BMPs are key players in adult stem cell biology.[380] They are involved in the control of the overall functional and phenotypic properties of the stem cell population (self-renewal, proliferation, differentiation, apoptosis, quiescence...). They can act directly on stem cells[380] or through their microenvironment,[50] contributing to the tight balance of this system. In the tumorigenic context, alterations of the BMP signaling pathway are involved in the deregulation of the interaction between stem cells and their microenvironment and, as such, participate to the different steps of the transformation process.[381,382] Targeted disruptions of murine BMP4, BMP2, BMPRIa or BMPRII all result in early embryonic lethality and reduced formation of mesoderm.[383–387] BMP

pathway also plays key roles during later development processes, especially during bone formation, cardiogenesis and hematopoiesis, as well as sustaining adult tissue homoeostasis.[330]

2. Stem cell regulation

At present, the involvement of the BMP pathway in SC biology is clearly established during the embryonic period and begins to be decoded in the adult, particularly in humans. Indeed, recent studies have demonstrated a key role for BMPs in the regulation of both SC and their microenvironment. BMP transduces a signal within SC, thus regulating their functional properties (self-renewal, proliferation, differentiation, apoptosis, quiescence...). These effects are especially more complex as they depend on the cellular context studied and the BMPs involved. Indeed, while BMP4 promotes the self-renewal of humans HSC in vivo,[388] which was demonstrated by its ability to increase the HSC repopulation activity in irradiated NOD/SCID immunodeficient mice, this protein has different effects in other SC types. For example, in the human nervous system, BMP4 stops neural SC (NSC) in G_0/G_1 phase of the cell-cycle.[389] This effect is reversible as the cells retain their SC properties. Maintenance of NSC quiescence involves the BMPRIa receptor and Smad4 in response to BMP2 and BMP4. Hence, BMPs control the SC functional properties depending on the cellular context, but also on the species involved. Indeed, in the murine system, BMP4 doesn't induce quiescence, but leads to NSC commitment to differentiation, with the loss of stem cell potential and the acquisition of mature cells properties.[390] BMP pathway is also able to maintain SC quiescence in mouse epithelial tissue, thus regulating follicular[391] and intestinal[392] SC self-renewal through control of the Wnt/beta-catenin pathway. BMP4 also promote the survival of SC and maintain their pluripotency in the mesenchymal system.[393] These examples illustrate the complexity of BMP signal specification, the biological resultant being the integration of many parameters. In the pancreas, BMP4 induces the proliferation and differentiation of the pluripotent AR42J rat pancreatic carcinoma cell line.[394] The injection of a BMP4 neutralizing antibody in $IFN\gamma^{-/-}$ NOD mice reduces the expansion of pancreatic progenitor cells. Thus, BMP4 seems to control both expansion and terminal differentiation of pancreatic progenitors. Finally, in the neural system, concentration gradients of BMP4 condition NSC engagement into different lineages,[395] suggesting a spatial regulation of BMP4 action on neurogenesis and a dependency towards environmental signals.[396] Thus, BMPs are conditional inducers of SC commitment towards specific lineages, whose final biological effects depend on the presence of specific cofactors.[397] BMP may thus maintain SC pluripotency, or, conversely, induce SC differentiation.

While the BMP signaling pathway seems to be involved in the control of SCs in most

adult tissues, it has been particularly well studied in the hematopoietic context, because of its importance in HSC fate control.

3. Hematopoiesis control

Microenvironmental signals control HSCs fate by activating some of their intrinsic signaling pathways. Several of them are conserved embryonic pathways including Wnt, Hedgehog, TGF-β, Notch, BMI1/polycomb[398] as well as BMP pathway. Multiple cell types within the BM niche produce BMPs, including stromal cells, megakaryocytes and platelets.[399] In addition, osteoblasts have been shown to secrete several BMPs, including BMP2, BMP4 and BMP7.[400,401] These molecules can then become incorporated into the ECM and act through an autocrine or paracrine signaling. BMP4 effect on hematopoietic development in the mouse has been established from of its genetic deletion, which disrupts mesoderm and blood cell formation in the yolk sac.[383] BMP2, BMP4, BMP7 and Smad proteins have been implicated in the self-renewal and maintenance of HSCs, expansion of progenitor cells and their differentiation into the mature lineages of the hematopoietic system.[388,402,403]

In 1999, it has been demonstrated that members of the BMP family regulate self-renewal, proliferation and differentiation of $CD34^+$, $CD38^-$, Lin^- HSCs extracted from human bone marrow and cord blood by combining *in-vitro* and *in-vivo* assays[388]. It was shown that high concentrations of BMP2 and BMP7 lead to an inhibition of the proliferation of $CD34^+$, $CD38^-$, Lin^- cells while maintaining their *in-vivo* repopulating capabilities along with phenotypic and functional properties of immature cells.[388] However, while at low concentrations BMP4 led to the proliferation and differentiation of these cells, at high concentrations, BMP4 was able to maintain their HSC phenotypic and functional properties.[388] This illustrate the primordial role of BMP pathway in HSC control as well as the complexity of this family of molecules which despite using the same signaling pathways can mediate different functional effects on a same cell population.

Our group later demonstrated direct involvement of BMP molecules in HSC differentiation. In the human hematopoietic system, BMP pathway was identified as a conditional inducer of human HSCs commitment, depending on the type of BMPs involved. Indeed, whereas BMP2 or activin A directed differentiation of human $CD34^+$ progenitors towards erythrocytic lineage in absence of EPO,[404] BMP4 favored their commitment towards megakaryocytic lineage (Figure 20).[399] This suggests that BMP2 and BMP4 do not share redundant biologic function in the regulation of hematopoiesis,[404] despite their high level of homology. However, these effects may also depend on the co-factors involved in these processes. Indeed, BMP4 was shown to induce erythrocytic commitment in cooperation with SCF and EPO,[405,406] while BMP4 alone induced megakaryocytic differentiation.[399]

Conversely, BMP2 do not seem to be involved in megakaryocytic differentiation.[399] Another study also demonstrated the role of Smad5 in BMP4 signaling as Smad5 inhibition in human hematopoietic progenitors lead to the inhibition of BMP4 mediated erythropoiesis.[407] Interestingly, it was recently demonstrated that Smad6, a BMP inhibitor, was capable to inhibit erythropoiesis in human cord blood HSCs by blocking Smad5-mediated BMP4 signaling in presence of EPO.[408] Thus, these data suggest that Smad6 indirectly maintains stemness by preventing spontaneous erythropoiesis in HSCs.[408]

Even if its role in hematopoiesis has not been extensively studied, BMP7 also seems to participate in the control of HSCs number.[409] Indeed, low-doses of BMP7 improved the proliferation of total cells, $CD34^+$ cells and $CD34^+$, $CD38^-$ cord blood cells without affecting the colony-forming ability or the engagement of $CD34^+$ cells. Moreover, cord blood-derived $CD34^+$ cells cultured with BMP7 for 10 days showed better engraftment and multi-lineage reconstitution ability after engraftment into irradiated NOD/SCID mice.[409]

Moreover, Smad5 has been shown to negatively regulate the proliferation and self-renewal of early yolk-sac-derived multipotent hematopoietic progenitors.[362,410] This was shown by $Smad5^{-/-}$ yolk sacs that gave rise to an increased number of granulocyte-macrophage colony-forming units by CFC assay. However, this effect was explained by a decreased sensitivity to TGF-β1 inhibition of hematopoiesis,[362] while the possibility for soluble BMPs involvement was not tested, even though they are the main upstream ligands of Smad5.

While the previous examples indicate direct regulation of HSCs by BMPs, their impact can also be indirect. Indeed, it has recently been demonstrated that BMP4 is able to induce integrin-α4 expression in immature human Lin^-, $CD34^+$ cells through Smad-independent pathway, thus increasing their homing and subsequent hematopoietic reconstitution capabilities in irradiated mice.[411]

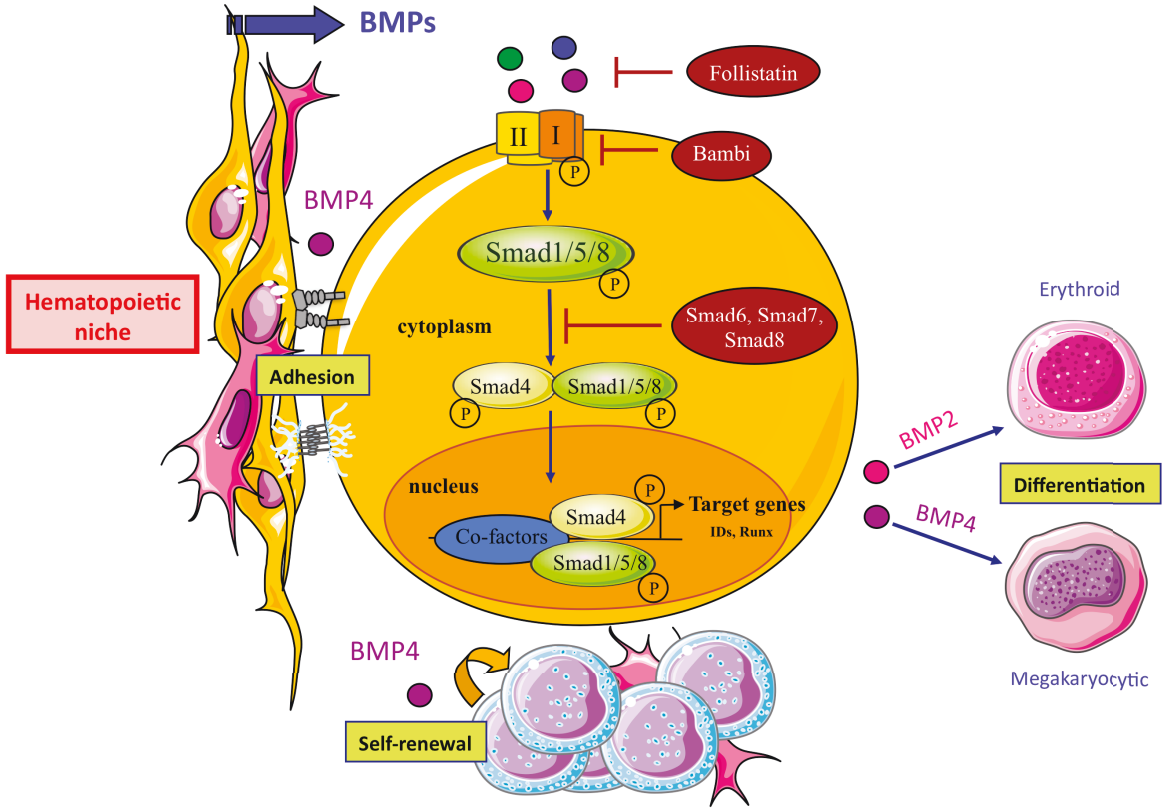

Figure 20 : Role of BMP2 and BMP4 in the regulation of hematopoietic stem cells fate. Soluble BMPs, such as BMP2 and BMP4 are produced by the bone marrow microenvironment, but also by hematopoietic cells themselves. These molecules will trigger the activation of the intrinsic BMP signaling pathway in HSCs, leading to different functional consequences, ranging from differentiation to self-renewal or even quiescence, depending on the stage of cell immaturity or the presence of additional co-factors.

Finally, our group demonstrated in 2006 that the BMP signaling pathway is also involved in the regulation of HSCs through the direct involvement of its soluble inhibitors follistatin and FLRG.[412] Indeed, the results obtained showed that follistatin and FLRG interact with fibronectin and that this interaction is accompanied by an increase in the adhesion of normal human hematopoietic progenitors to fibronectin.[412] Interestingly, HSC adhesion to the BM stroma has been demonstrated to decrease their proliferation[39] and to induce quiescence,[413] mechanism probably involved in CML LSC resistance to TKIs.[187] Hence, BMP pathway could also be involved in the regulation of HSCs fate through control of their interactions with their BM microenvironment.

V. Microenvironment control

1. Physiological context

One of the major difficulties to analyze BMP effects on SC biology lies in the multiplicity of biological effects often seemingly contradictory. The biological effects are conditioned both by intrinsic parameters (signaling pathways elements) and extrinsic parameters in a specific environment (co-factors and soluble BMPs production). Thus, from embryogenesis to adulthood, BMPs act under the control of the cellular microenvironment in multiple systems. Whatever the studied system, SCs reside in niches consisting of a multitude of cell types that synthesize compounds such as BMP proteins. These, in turn, are able to act on the different elements and actors of the niche and will participate in the control of SC fate. Indeed, BMPs distribution and action in time and space are dictated by the niche itself (size and composition), whose elements are also under the control of the BMP pathway.[50] For example, BMPs have been described as key regulators of mesenchymal SC (MSC) differentiation toward different lineages: adipocytes, osteogenic or chondrogenic.[414,415] The dialogue between SCs and their niche thus creates a perpetual, dynamic and reciprocal regulatory network. Thus, the balance between these different parameters allows a fine control of SC biology in their microenvironment.

The use of BMPRIa deficient mice has, for first time, demonstrated the proof of the indirect action of the BMP pathway on HSCs through control of the hematopoietic niche. Indeed, the increased number of osteoblasts in the bone marrow of BMPRIa$^{-/-}$ mice led to a significant expansion of HSCs in these animals.[50] This study evidenced that the BMP signaling controls the hematopoietic niche size through regulation of osteoblast production, which will act as a support for HSC amplification. Thus, this study remarkably demonstrated that alterations of the BMP signaling pathway can be involved in abnormal proliferation of different cell types such as osteoblasts or HSCs, and possibly in tumorigenic processes.

Interestingly, another study demonstrated the crucial role of BMP4 in the maintenance of a competent HSC niche.[416] Indeed, serial transplantation studies reveal that BMP4-deficient recipient mice have a microenvironmental defect that reduces the repopulating activity of wild-type HSCs. Furthermore, wild-type HSCs that successfully engrafted into BMP4-deficient recipient mice show a marked decrease in functional HSC activity when tested in a competitive repopulation assay.[416] Even though the exact mechanism is still unknown, as BMP4 is able to induce HSCs homing through control of integrin-α4 expression,[411] the loss of BMP4 in the BM microenvironment could impair HSC homing, consequently leading to the progressive loss of their HSC properties.

2. Tumoral context

At present, many studies suggest that the CSC microenvironment could be altered without being formally transformed. In leukemias, some studies suggest that the alterations could affect both cell populations present in this microenvironment and the components of the ECM such as integrins, cytokines, fibronectin or Wnt pathway ligands. The alteration of the microenvironment has also been reported in solid tumors, leading to the disruption of collagen and fibronectin fibers, the clustering of integrins and the abnormal activation of different signaling pathways.[307,310,417,418]

The majority of hematological malignancies are characterized by more or less marked alterations of the bone marrow composition, with often myelofibrosis that increases with disease progression.[419] Yet, BMPs are known for their involvement in osteogenic processes.[324,325] Hence, an alteration of the BMP pathway, embodied for example by an alteration of the soluble BMPs concentrations in the bone marrow, could be involved in this myelofibrosis.[420] Interestingly, it has been demonstrated that Imatinib, the first-line treatment of CML, increases the transcriptional expression of BMP2 in human MSCs[421] and is able restore a consistent bone marrow structure.[422]

Moreover, multiple myeloma (MM) is characterized by a progressive destruction of the bone tissue due to enhanced osteoclastogenesis and suppressed osteoblastogenesis.[423] A recent study revealed that Pim-2, a kinase involved in tumor progression and bone loss in MM, is able to abrogate BMP2-induced osteoblastogenesis *in-vitro*. This highlight the crucial role for BMP pathway control in the regulation of the BM niche, and illustrates how alterations of this pathway can lead to alterations of the BM microenvironment, thus participating in tumor progression.[424]

Finally, the importance of the BMP pathway in the control of SC microenvironment has also been observed in other tissues. Interestingly, our group recently evidenced that BMP alterations in the breast microenvironment are involved in early steps of SC transformation.[425] Specifically, it has been demonstrated that BMP2 production by tumor microenvironment is up-regulated in luminal tumors. While BMP2 is involved in the control of early luminal progenitors maintenance and differentiation under physiological conditions, chronic exposure of immature human mammary epithelial cells to high BMP2 levels initiated transformation toward a luminal tumor-like phenotype. It was further demonstrated that BMP2 signaling was mediated by BMPRIb receptor. This study also suggests that microenvironment-induced over-expression of BMP2 may result from carcinogenic exposure, thus providing new evidences for the role of microenvironment alterations in tumorigenic processes through altered BMP signaling.[425]

VI. BMP signaling pathway, cancer and cancer stem cells

BMP signaling pathway is involved in different pathological processes such as cancer[381] or osteoporosis,[426] but also in conditions affecting the heart, lungs, eyes and reproductive organs.[330] The role of the BMP family in cancer processes has been particularly well documented. The diversity of cancer types concerned demonstrates the importance and pervasiveness of this signaling pathway in tumorigenic processes and cancer dissemination. These observations are sometimes used clinically as prognostic factors or for the identification and classification of some tumors. This is the case for example in prostate cancer where the loss of BMPRII expression is a good prognostic factor.[427] BMP2 expression constitutes a factor of poor prognosis in gliomas[428] and could be associated with tumor progression in gastric cancer.[429] The expression of membrane BAMBI inhibitor also seems to be suppressed by epigenetic modification in a subtype of high-grade prostate cancer.[430]

While the major role of the BMP pathway in oncogenic processes is undeniable, it is only very recently that its involvement in CSC biology was suspected.[382] BMPs are key regulators of normal SC biology and recent studies have started to demonstrate their role in CSC biology. In glioblastoma, BMPRIb has been involved in the maintenance of the CSC phenotype.[358] Indeed, in the tumor, there is a refractory cellular fraction to the BMP4-induced differentiation that persists and that is able to promote tumor reconstitution. In this CSC subpopulation, BMPRIb expression is quenched by methylation of its promoter and its re-expression in response to a demethylating agent can restore CSC differentiation under BMP4 treatment.[358] Treatment of neural CSC by BMP4 decreases their proliferation without affecting their viability, induces their differentiation into neural precursors and reduces their ability to form tumors in NOD/SCID mice. Thus, BMP4 reduces the neural CSC pool by pushing them towards a differentiation process and therefore constitutes a potential therapeutic agent in this very aggressive disease.[431] However, while BMPs are highly expressed in glioblastomas (particularly BMP2 produced by CSC, but also BMP7 produced by adjacent NSC), they do not manage to induce CSCs differentiation *in-vivo*. Interestingly, a recent study demonstrated that Gremlin1, a BMP antagonist, is secreted by CSCs[432] and is able to protect them against the BMP-induced differentiation.[433]

In melanoma, the existence of CSC was evidenced in humans in 2006.[434] These CSC express specific markers such as ABCB5 (ATP-binding cassette sub-family B member 5) and over-express BMPRIa.[435] However, the functional role of the BMP pathway in melanoma CSC has not been described yet.

Our group recently demonstrated that BMP alterations in the breast microenvironment are involved in early steps of SC transformation.[425] However, alterations of the BMP pathway

were also observed in tumor cells themselves, as an increased BMPRIb expression was observed in luminal tumor cells. BMPRIb expression was increased after chronic exposure to BMP2, and was further shown to be required for BMP2-induced transformation of immature mammary epithelial cells.[425]

VII. BMP signaling pathway in leukemia

BMP signaling pathway is deregulated in various bone-related disorders and cancers, including acute promyelocytic leukemia (APL or AML M3). Indeed, BMP2, BMP4, BMP7, as well as the receptors BMPRIa, BMPRIb and BMPRII, were shown to be up-regulated in APL leukemic blasts, with levels returning to normal in patients who responded to all-trans-retinoic acid (ATRA) treatment.[436] In patients who did not respond to ATRA therapy, BMP elements expression remained high in the malignant promyelocytes. This study highlights the potential for the use of the BMP pathway as a minimal residual disease molecular marker in APL.[436] Interestingly, another study assessed the role of BMPs in the response of AML M3 cells to ATRA treatment. It was demonstrated that BMP2, BMP4 and BMP6 were able to block ATRA-mediated differentiation of leukemic promyelocytes by inducing the expression of inhibitor of differentiation (ID) genes.[437] These results further suggest the possible use of BMP antagonists as complementary therapeutic strategy for ATRA-resistant patients.

Furthermore, it has recently been demonstrated that expression of CBFA2T3-GLIS2 fusion protein in a small subset of acute megakaryoblastic leukemia (AMKL)[438] patients was able to increase the self-renewal capability of committed myeloid progenitors along with megakaryocytic differentiation, in part through enhanced BMP2 and BMP4 signaling.[439]

Mesoderm Inducer in Xenopus Like1 (MIXL1), a homeobox transcription factor aberrantly expressed in AML and lymphomas was identified for its leukemogenic potential both *in-vitro* and *in-vivo*. Interestingly, a recent study demonstrated that BMP4 is able to induce MIXL1 expression in human HSCs.[440] The anti-apoptotic advantage conferred by an increased MIXL1 expression was reversed upon type I BMP receptor inhibition, suggesting the BMP pathway as a potential target for therapy. Interestingly, MSX1, another homeobox transcription factor, was found to be over-expressed in 11% of T-ALL patients. In that case, it was demonstrated that the increased MSX1 expression was due to the inhibition of BMP4 signaling by chordin-like1 (CHRDL1) inhibitor.[441]

These results illustrate the duality of the BMP pathway that can either repress or activate tumorigenic processes. This is further supported by recent studies demonstrating that BMP4 and BMP6 are able to induce apoptosis of multiple myeloma (MM) cells by Smad-dependent repression of MYC[424,442]. However, the exact role of BMP signaling pathway in leukemic maintenance and progression is still not clearly defined.

These results demonstrate that BMP signals are crucial for SC regulation, particularly in the hematopoietic context where BMPs control HSC and their microenvironment. In addition, BMP signaling is altered in most cancer processes including leukemias, and seems involved in CSC phenotype. Finally, BMPs have been described as key regulators of mechanical signals transduction, process also involved in HSC regulation and tumorigenic processes. Altogether, these results support a potential role for BMP signaling in CML LSC biology, potentially mediated by mechanical signals.

Aims

I. Research problem

One of the main causes of treatment failure in cancers is the development of drug resistance by cancer cells. The persistence of CSC might explain cancer relapses as they could allow reactivation of cancer cells proliferation following therapy, leading to disease persistence and ultimately to patients' death. Clinically, it is crucial to develop therapeutic strategies able to target resistant CSC in order to cure the patients. In this context, CML is the reference model of a true HSC alteration by a t(9,22) chromosomal translocation leading to the formation of the BCR-ABL fusion protein, with high tyrosine kinase activity and directly responsible of the leukemic transformation. Despite the success of targeted therapies against BCR-ABL with the development of TKIs, a residual disease is still detected in many CML patients, probably due to the persistence in vivo of CML leukemic stem cells (LSCs). In some cases, resistance to TKI treatment appears, involving different mechanisms with 30% of unknown origin that could be due to LSCs survival. LSCs are controlled by a variety of biochemical and biomechanical signals from the leukemic niche. Thus, deregulation of these signals could contribute to LSC emergence and resistance. My project aims to determine the involvement of the tumor microenvironment (BMP signaling pathway and mechanical stress) in the maintenance and resistance of CML LSCs.

II. Scientific context

BMP pathway is part of the TGF-β super-family signaling pathway. This signaling pathway is crucial for the regulation of HSCs and their microenvironment (Chapter 4). Interestingly, TGF-β signaling has been demonstrated to potently inhibit the growth of BCR-ABL-expressing $CD34^+$ cells isolated from CP-CML patients by inducing cell-cycle arrest.[443] It was further reported that inhibition of the TGF-β receptor kinase in a quiescent sub-population of CML $CD34^+$ cells is able to overcome the cell-cycle blockade induced by TGF-β, and to enhance their sensitivity to Imatinib treatment.[443] However, these results are probably cell type dependent, as another report suggested that BCR-ABL is able to inhibit the cytostatic effect of TGF-β through the AKT/FoxO3 signaling pathway in Ba/F3 and K562 cells.[444] While its role in normal HSCs control is undeniable, the BMP pathway has not been extensively studied in the CML context. However, recent studies raised very interesting mechanisms supporting the importance of this signaling pathway in CML. For example, the impact of Imatinib treatment on human bone marrow mesenchymal stem cell differentiation has recently been assessed *in-vitro*.[421] It has been demonstrated that Imatinib, the first-line treatment for CML, was able to induce mesenchymal stem cell differentiation toward the osteoblastic lineage, together with an increase in osteoblastic markers such as BMP2 and Runx2.[421] Thus, Imatinib treatment could have a direct impact on BMP production in BM microenvironment, leading to the alteration of BMP signaling in healthy and CML immature cells. Moreover, as discussed in this study, this could lead to an alteration in the cellular composition in the BM niche, affecting others mechanisms involved in HSCs regulation. This is further supported by the study previously discussed, demonstrating that BMP signaling is able to control hematopoietic niche size through regulation of osteoblast production, which acts as a support for HSC amplification.[50] Although this has not yet be reported in the literature, some evidences advocate for a potential role of the BMP pathway in CML phenotype. For example, Twist-1, an embryonic transcription factor recently identified as a new predictive factor for the effect of TKIs on CML cells,[445] can regulate or be regulated by the BMP pathway, depending on the cellular context.[446–449] BMP signaling is also altered in different types of cancers[381] including leukemia and is involved in CSC properties.[382] CML is characterized by the alteration of the bone marrow structure with disease progression,[419] and BMPs have first been identified for their role in regulating bone morphogenesis.[324,325] CML arises from the alteration of a HSC, and BMPs are key regulators of hematopoietic stem cells.[50,388] Finally, CML is also characterized by the alteration of hematopoiesis, particularly of myelopoiesis,[450] and BMPs are known regulators of hematopoietic stem cells commitment (Figure 21).[399,404]

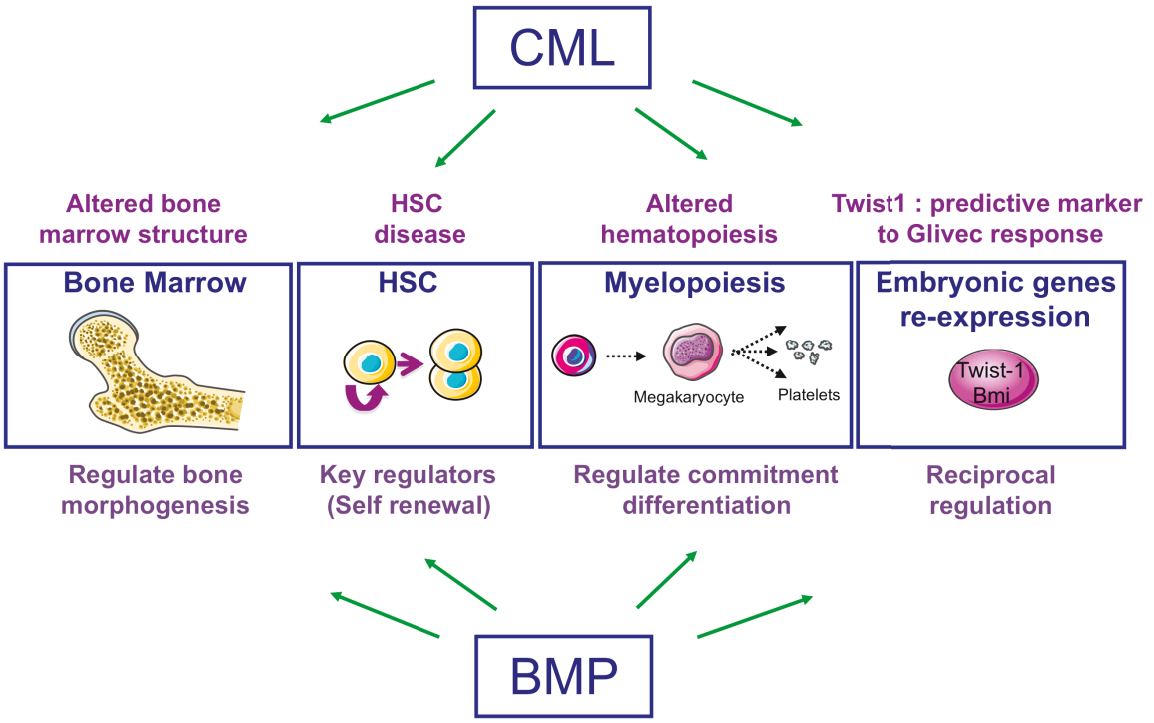

Figure 21 : BMP pathway and Chronic Myelogenous Leukemia. CML is characterized by the alteration of the bone marrow structure with disease progression, and BMPs have first been identified for their role in regulating bone morphogenesis. CML arises from the alteration of a hematopoietic stem cell, and BMPs are key regulators of hematopoietic stem cells. CML is also characterized by the alteration of hematopoiesis, particularly of myelopoiesis, and BMPs are known regulators of hematopoietic stem cells commitment. Finally, some embryonic genes are re-expressed in CML such as *Twist-1*, gene that control or that can be controlled by BMP pathway depending on the cellular context.

Interestingly, BMP pathway is a key regulator of mechanical signals transduction in cells (Chapter 4), that are also involved in SCs regulation (Chapter 3). Moreover, recent studies have demonstrated the role of cell mechanics in HSCs regulation. Due to their faculty to circulate through blood vessels, hematopoietic cells experience a wide variety of dynamic forces. Thus, their viscoelastic response under fluid shear stress and deformation have been extensively studied. Variations in blood viscoelasticity have been observed in conditions such as cardiovascular disease, peripheral vascular disease, sickle cell anemia, diabetes and stroke.[451,452] Bone marrow provides a number of distinct mechanical environments associated with sinusoids, blood vessels, and mineralized bone matrix. Several of these regions are complex but well characterized niches comprised of osteocytes, osteoblasts, osteoclasts, endothelial cells, mesenchymal stromal cells, as well as mature blood lineages such as macrophages.[452] Thus, HSCs can experience a wide array of elasticities in the BM, from under 1 kPa for adipose tissue to 100 kPa for collagenous bone.[452] However, the role of physical signals in HSCs fate control just begins to be deciphered. Interestingly, a recent study revealed for the first time the critical role of biomechanical forces in hematopoietic

development.[46] Using mouse embryonic SC differentiated *in-vitro*, it has been demonstrated that fluid shear stress increases the expression of Runx1, a master regulator of hematopoiesis,[453] in $CD41^+$, c-Kit^+ hematopoietic progenitor cells, concomitantly increasing their hematopoietic colony-forming potential. It was further demonstrated that abrogation of nitric oxide, a mediator of shear-stress-induced signaling, compromises hematopoietic potential *in-vitro* and *in-vivo*.[454] In addition, another study suggested the importance of substrate elasticity in the control of hematopoietic stem and progenitor cell fate.[455] Indeed, culturing mouse Lin^-, Sca^-1^+, c-Kit^+ or human Lin^-, $CD34^+$, $CD38^+$ immature hematopoietic cells on a tropoelastin substrate led to a two- to three-fold expansion of undifferentiated cells, including progenitors and mouse stem cells. Treatment with cytokines in the presence of tropoelastin had an additive effect on this expansion. While an increase in cell forming colony (CFC) content was observed in cells cultured on tropoelastin-coated plates compared to controls, no difference in the size or type of colonies was observed.[455] Mice HSCs were then cultured on control or tropoelastin-coated plates and transplanted into mice, demonstrating a higher repopulating capabilities after culture on tropoelastin-coated plates. These biological effects required substrate elasticity, as neither truncated nor cross-linked tropoelastin proteins could reproduce the phenomenon, and inhibition of mechano-transduction signals abrogated these effects.[455] These evidences support the use of optimal physical substrates that may complement existing approaches to support and expand HSCs. As a crucial role for cell mechanics was recently evidenced in HSCs fate control, mechanical signals could also be involved in pathologic processes such as leukemia, where these signals are likely to be altered. Due to its confinement by bones, the bone marrow has a unique mechanical environment that can be affected by external factors, such as blood flow, physiological activity, aging or disease.[452] This could prove especially important in the tumoral context, where uncontrolled proliferation of leukemic cells entails their compaction within their microenvironment, resulting in constant mechanical stress (Figure 22).[316] Moreover, in CML, Imatinib treatment has been demonstrated to re-establish a coherent medullar structure in treated patients (Figure 22).[422] Thus, changes in the bone marrow mechanical environment during the course of the disease may also affect the fate of resident HSCs or LSCs. Finally, alterations of intrinsic mechano-transduction pathway in leukemic cells could impair their capability to sense and/or respond to mechanical signals.

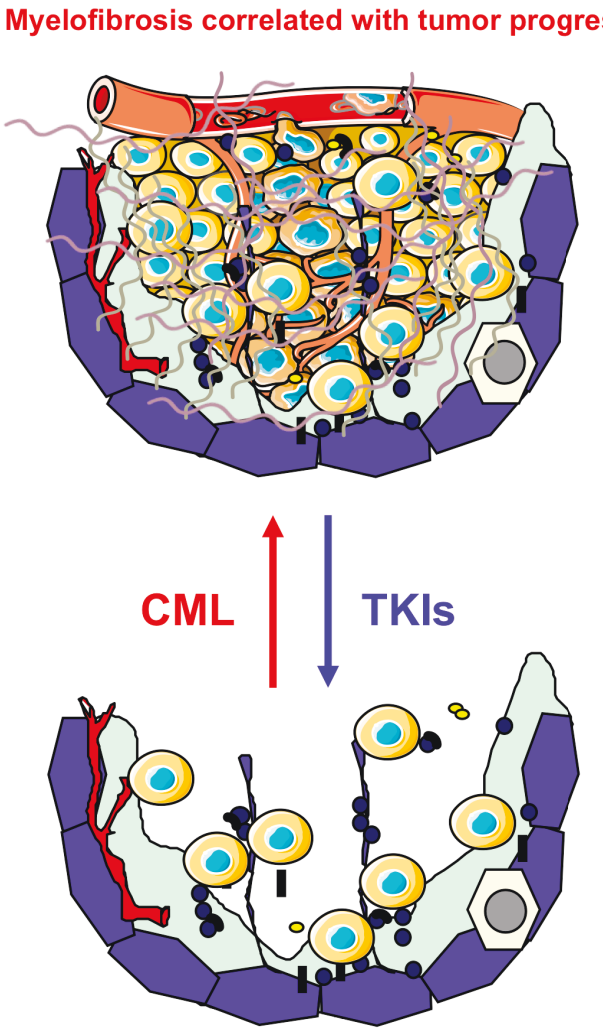

Figure 22 : Possible alterations of the mechanical signals in the CML bone marrow environment. Like in most hemopathies, CML is often characterized by a hyper-cellularity in the bone marrow, eventually leading to the alteration of the bone marrow structure, with the appearance of myelofibrosis in some cases, which is accentuated with disease progression. Imatinib treatment has been demonstrated to re-establish a coherent medullar structure in CML patients. These differences in cellularity as well as in bone marrow structure could possibly lead to an alteration of the mechanicals signals provided by the microenvironment to HSCs and LSCs.

Given the crucial role of both BMP signaling and mechanical signals in HSC regulation, and their involvement in cancer, it is reasonable to speculate that alteration of these processes could be involved in the leukemic phenotype in CML.

III. Thesis project

My project aims to determine the involvement of the tumor microenvironment in maintenance and resistance of CML LSC. For that, we first assessed the potential role of BMP signaling in immature CP-CML cells by combining functional and molecular assays to the analysis of tumor microenvironment in more than 70 CP-CML patient samples at diagnosis without any treatment. We then evaluated whether BMP pathway could be involved in immature CML cells resistance to TKIs treatment by performing resistance assays and analyzing sensitive and resistant CML patient samples (at diagnosis, resistance status obtained after clinical follow-up of at least 2 years). In parallel, we benefited from the expertise of the physics laboratory in microsystems design and mechanical measurements to understand how mechanical stress exerted by the microenvironment could influence immature CML cells fate. We used atomic force microscopy to compare the mechanical properties of single living $CD34^+$ normal or CML cells. Finally, we used an innovative system allowing a defined confinement of *in-vitro* cultured cells to evaluate the impact of external mechanical stress on $CD34^+$ CML cells functional properties as well as their response to TKIs. We hope that this transdisciplinary approach will help to identify key molecules in the transduction of signals potentially involved in maintenance and resistance of LSCs and thus offer new targets to counter these effects.

Material and methods

Materials

I. Human primary cells

After informed consent in accordance with the Declaration of Helsinki and local ethics committee bylaws (from the Délégation à la recherche clinique des Hospices Civils de Lyon, Lyon, France), peripheral blood and bone marrow samples were obtained from CML patients at diagnosis or during follow-up and from healthy allogeneic donors. Mononuclear cells were separated using a Ficoll gradient (Bio-Whittaker) and were then subjected to CD34 immunomagnetic separation (Stemcell Technologies). The purity of the $CD34^+$ enriched fraction was checked by flow cytometry and was over 95% on average (Figure 23). Selected bulk $CD34^+$ cells were seeded at $6x10^5$ cells/ml and cultured in serum-free Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen) in the presence of 15% BSA, Insulin and Transferrin (BIT) (Stemcell Technologies) supplemented with 10 ng/ml interleukin-6 (IL-6), 50 ng/ml stem cell factor (SCF), 10 ng/ml IL-11 and 10 ng/ml IL-3 (Peprotech). Stromal cells were isolated by plating bone marrow samples from healthy donors or CML patients at $5x10^4$ cells/cm^2 in Alpha Modified Eagle's Medium (α-MEM) (Invitrogen) supplemented with 10% Fetal Calf Serum (FCS) and culturing them for 2 to 3 weeks. Cells were allowed to attach for 7 days, then any non-adherent cells were carefully removed and fresh medium was added. When the primary cultures became almost confluent, remaining stromal cells were washed twice in PBS1X and harvested using trypsin-EDTA (Life Technologies).

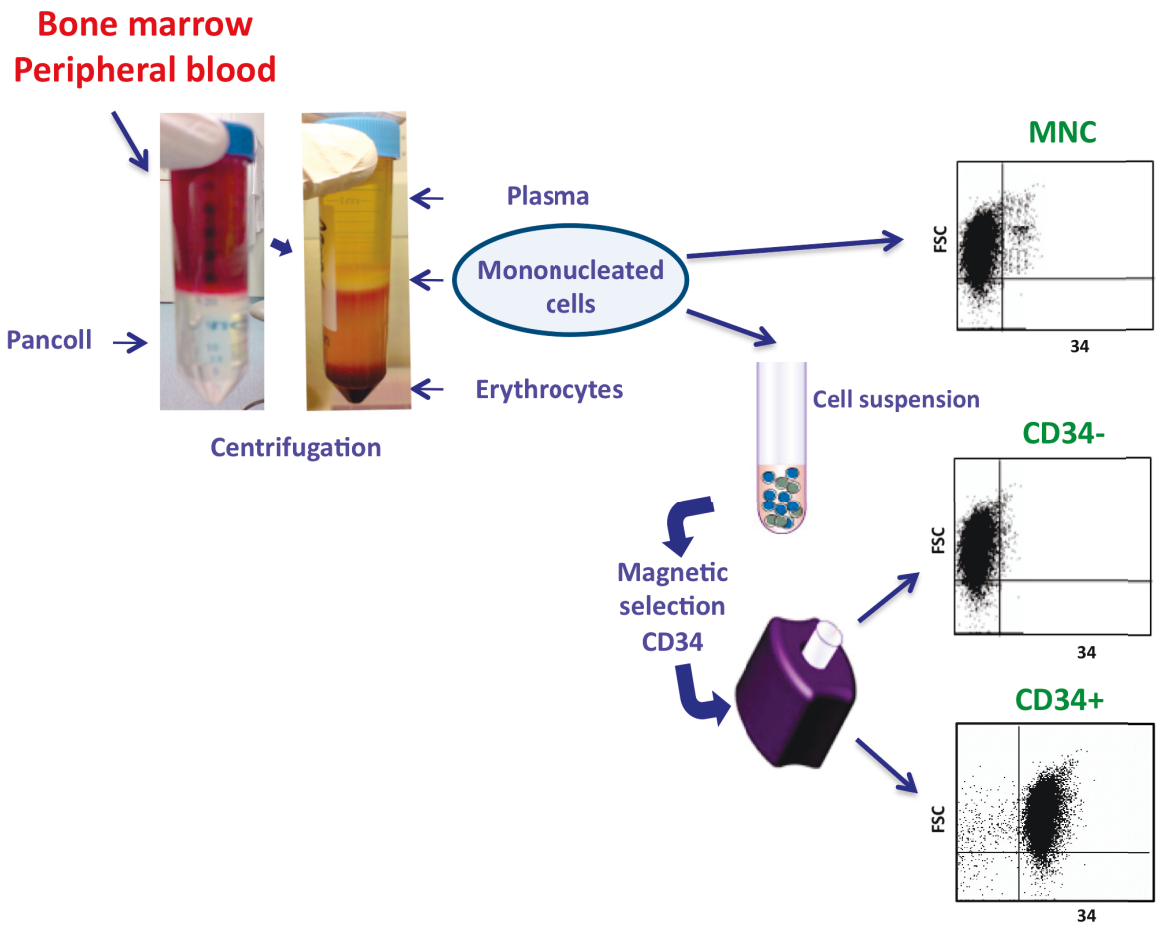

Figure 23 : Primary samples process. Mononuclear cells were separated using a Pancoll gradient (Bio-Whittaker) and were then subjected to CD34 immunomagnetic separation. Before the gradient separation and for bone marrow samples only, the plasma was harvested and $5x10^4$ cells/cm^2 were plated in α-MEM medium to culture stromal cells.

II. Cell lines

The BCR-ABL$^-$ hematopoietic cell line TF-1 (ATCC CRL-2003) has been derived from a patient with erythroleukaemia and was maintained at $1x10^5$ cells/mL in Roswell Park Memorial Institute (RPMI-1640) medium (Invitrogen), 10% FCS and granulocyte macrophage colony-stimulating factor (GM-CSF, 10 ng/mL) (Sandoz Pharmaceuticals). Engineered TF1-GFP and TF1-BCR-ABL-GFP cell lines were obtained by transduction with an MSCV-based retroviral vector[456] encoding either the enhanced green fluorescent protein cDNA alone (EGFP) as a control or the BCR/ABL-cDNA upstream from an IRES-eGFP sequence (Figure 24). EGFP$^+$ TF1 cells were sorted using a Becton Dickinson FACSAria. The BCR-ABL$^+$ hematopoietic cell line KCL22 (ATCC CRL-2003) has been established from the pleural effusion of a CML patient in blast crisis and was maintained at $1x10^5$ cells/mL in RPMI-1640 medium (Invitrogen) and 10% FCS.[457] The stromal cell line HS27A (ATCC CRL-2496) has

been derived from a human bone marrow and was maintained at $1x10^4$ cells/mL in RPMI-1640 medium (Invitrogen) and 10% FCS. The stromal cell line HS5 (ATCC CRL-11882) has been derived from a human bone marrow and was maintained at $1x10^4$ cells/mL in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) and 10% FCS. The microvascular endothelial cell line BMEC-1 has been derived from a human bone marrow and was maintained at $1x10^4$ cells/mL in MCDB-131 medium (Invitrogen) and 10% FCS. The murine stromal cell line MS5 (kind gift from Dr. Coulombel, Creative Bioarray CSC-C2763) has been derived from murine bone marrow and was grown in α-MEM (Invitrogen) supplemented with 10% FCS at $0,5x10^4$ cells/ml. MS5 cell line was used as feeder to support human hematopoiesis in LT-CIC assays. Hematopoietic cell lines were passages twice a week while stromal and endothelial cell lines were passaged every 2 weeks.

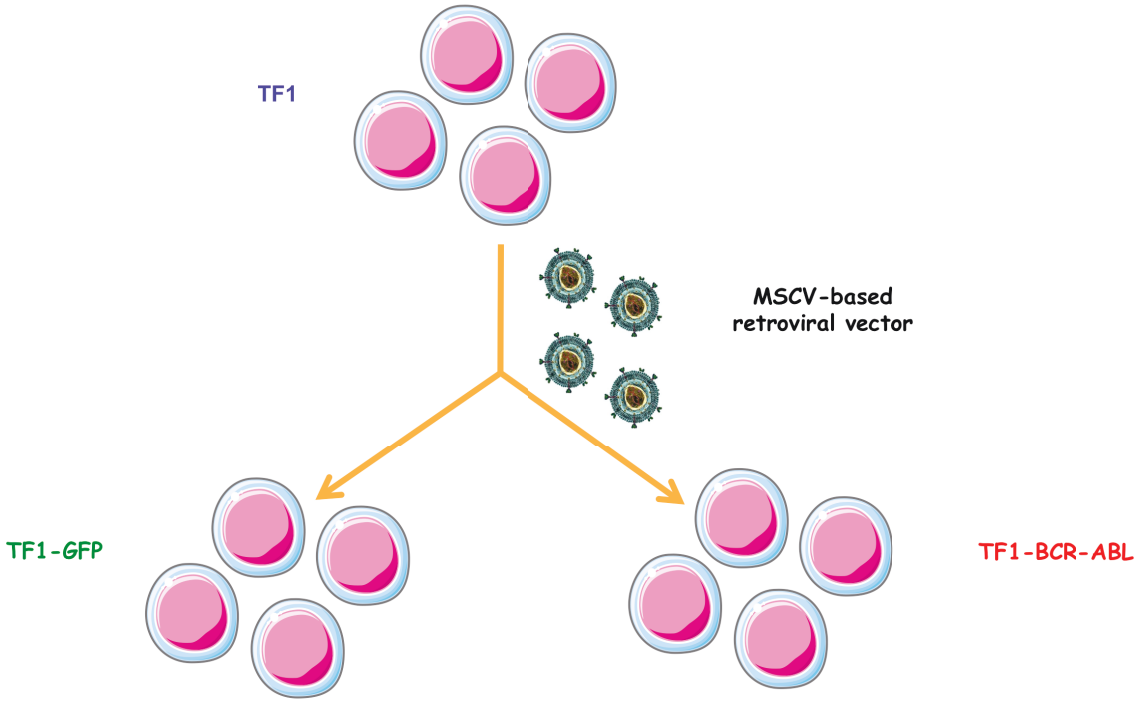

Figure 24 : Engineered BCR-ABL$^+$ CD34$^+$ TF1 cells. Engineered TF1-GFP and TF1-BCR-ABL-GFP cell lines were obtained by transduction of the human CD34$^+$ TF1 cell line with an MSCV-based retroviral vector encoding either the enhanced green fluorescent protein cDNA alone (EGFP) as a control or the BCR/ABL-cDNA upstream from an IRES-eGFP sequence. EGFP$^+$ TF1 cells were sorted using a Becton Dickinson FACSAria.

III. Soluble molecules

BMP2, BMP4 (50 ng/ml) (R&D Systems), sBMPRIa and sBMPRIb (4 µg/ml) (R&D Systems) were added as determined[399].

IV. Microfabricated wells

Microfabricated wells used in AFM setup for mechanical immobilization of suspended hematopoietic cells were created as described (Biotray).[298] Wells were patterned in arrays with diameters ranging from 15 to 20 µm. No differences in cell morphology or viability were observed between cells incubated on epoxy-based negative photoresist SU-8 and glass surfaces.

V. Microfabricated structured slides

Structured confining slides used for cell confinement were created in Polydimethilsiloxane (PDMS, RTV615, GE) from molds fabricated by regular photolithography (Biotray) as described.[273,458–460] For fixed height confining slides, one layer of epoxy-based negative photoresist SU-8 with the desired thickness was used (the thickness of the layer corresponding to the confinement gap: 30 µm, 10 µm, 5 µm and 2.5 µm). To fabricate the micro-structured glass slide, a drop of PDMS mix (8/1 w/w PDMS/crosslinker) was first poured on the mold. Then, a standard microscope coverslip (14 mm for 24-well plate experiments) previously activated for 2 minutes in a UV-ozone cleaner, was pressed onto the PDMS drop to get a residual PDMS layer of minimal thickness. After reticulation of PDMS on a hot plate (95°C, 15 minutes), the excess of PDMS was removed. The slide was gently lifted by inserting a razor blade between the slide and the mold. The surface of the glass slide covered by the microstructured PDMS layer was cleaned in isopropanol. Slides were sterilized under UV and incubated in the culture medium for 2 hours prior to the experiment. No differences in cell morphology or viability were observed between cells incubated on PDMS or plastic surfaces.

Methods

I. Functional assays

1. Cell counting and trypan blue dye exclusion

The number of viable cells was obtained following trypan blue staining using a Malassez counting chamber. The proliferation rate was represented as the ratio of counts at the end of the experiment to the number of input cells.

2. Cell adhesion assay

Adhesion was performed as described.[412] Briefly, fibronectin (Sigma) was coated for 2

hours at 37°C on 96-well Cellstar dishes (Greiner) using a 50 µg/ml ligand solution in sodium bicarbonate buffer (0.1 mM). Plates were blocked with 0.3% BSA in PBS1X for 1 hour at 37°C. Cells (5.10^4/well) were labeled for 20 minutes at 37°C with 5 µM calcein-AM (Molecular Probes) in RMPI medium containing 0.1% BSA (without phenol red). Cells were then allowed to adhere to the coated plates in triplicate wells for 1 hour at 37°C in the adhesion buffer (PBS1X with 0.03% BSA). The total fluorescence was quantified using a fluorescence analyzer (Cytofluor, PerSeptive Biosystems). Non-adherent cells were then removed, and after several gentle washes, the fluorescence of the adherent fraction was quantified. Adhesion was calculated as follow: % of adhesion = 100.(fluorescence of adherent fraction/fluorescence of whole cells).

3. Co-culture experiments

HS27A cells (ATCC CRL-2496) were seeded at $2x10^4$ cells/mL in Alpha Modified Eagle Medium (αMEM) (Invitrogen) supplemented with 10% FCS for 24 hours. The culture medium was then removed and selected CP-CML CD34+ cells were added at $6x10^5$ cells/mL and cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen), 15% BSA, Insulin and Transferrin (BIT) (Stemcell Technologies) supplemented with IL-6 (10 ng/mL), Stem Cell Factor (SCF) (50 ng/mL), IL-11 (10 ng/mL) and IL-3 (10 ng/mL) (Peprotech) for 7 days.

4. CFC assay

The content of treated cells in distinct hematopoietic progenitor categories was determined using the colony forming cell (CFC) assay. The assay is based on the ability of hematopoietic progenitors to proliferate and differentiate into colonies in a semi-solid media in response to cytokine stimulation. The cells were plated in Iscove's methylcellulose medium (Stem Cell Technologies) containing erythropoietin (EPO), SCF, IL-6, GM-CSF, G-CSF and IL-3 (Stem Cell Technologies) in 35-mm Petri dishes. After 15 days of incubation at 37°C, the colonies formed were enumerated and characterized in situ according to their unique morphology (Figure 25). We scored them as early erythroid (over three clusters), early myeloid (over 10^3 cells/colony), late erythroid (one to two clusters), late myeloid (below 10^3 cells/colony) as well as mixed colonies.

5. LTC-IC assay

The content of treated cells in hematopoietic stem cells was determined using the long-term culture-initiating cell (LTC-IC) assay. LTC-IC are a distinct, rare primitive hematopoietic cell type mostly found in bone marrow, which generate clonogenic cell progeny detectable after a minimum of 5 weeks incubation on suitable feeder cells layers. A high proportion of the $CD34^+$, $CD38^-$ cells in normal human marrow are defined as LTC-IC

because they can proliferate and differentiate when co-cultured with cytokine-producing stromal feeder layers. As long-term culture initiating ability is considered to be analogous to reconstitution of hematopoiesis in irradiated whole animals, determination of LTC-IC is thought to be a good measure of true pluripotent hematopoietic stem cells. The cells were co-cultured with murine MS5 cell feeder in human long-term culture medium (StemCell Technologies) supplemented with freshly dissolved 10 μM hydrocortisone sodium hemisuccinate (Sigma-Aldrich), with weekly half-medium change. After 5 weeks, both non-adherent and adherent cells were harvested, pooled, and washed; and the number of CFC produced was expressed as W5 CFC per 1000 initial $CD34^+$ cells and correlated with the number of primitive progenitors in the original input suspension (Figure 25).

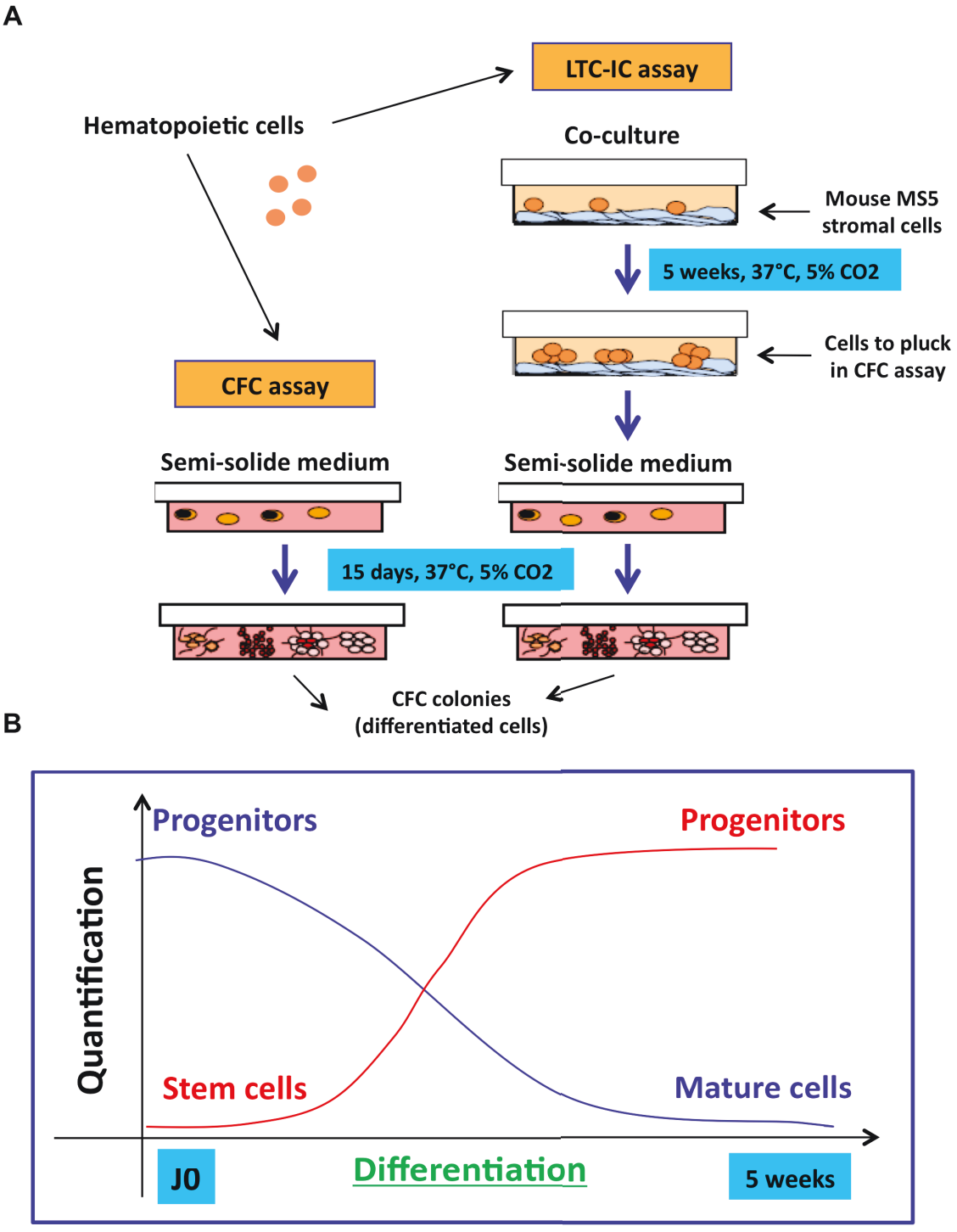

Figure 25 : Quantification of hematopoietic stem cells and progenitors. (A) CFC assay allow to quantify the content of a cell population in distinct hematopoietic progenitor categories. LTC-IC assay allow to quantify the content of a cell population in HSCs. (B) Cell content evolution during the 5 weeks of co-culture in the LTC-IC assay. During this period, the progenitors present in the starting suspension gradually differentiate into mature cells, lose their ability to divide and eventually disappear (blue curve). In contrast, HCSs differentiate into dividing progenitors and increase their number (red curve). Finally, CFC assay is performed after 5 weeks of co-culture to quantify the progenitor content, representative of the number of HSCs in the starting cell suspension.

II. Molecular assays

1. RNA isolation and analysis

Total cellular RNA was isolated from samples lysed in TRI-REAGENT (Sigma-Aldrich). For single CFC colony analysis, RNA extraction was performed using an RNA carrier (Glycoblue, Invitrogen). As indicated, 1 µg total RNA were reverse-transcribed with Superscript II enzyme (Invitrogen), and qPCR was performed using sequence-specific primers (Supplemental Table 1) using the QuantiFAST SyBR Green I kit (Qiagen) on the LightCycler 480 II system (Roche Applied Science) using a fast protocol (5 minutes at 95°C, 10 seconds at 95°C, 30 seconds at 60°C, 10 seconds at 50°C). Samples were analyzed in duplicate and normalized to TBP and BGUS gene expression previously selected by geNorm analysis. For all samples analyzed, results were validated and normalized using the same $CD34^-$ healthy-donor cells used as a reference.

2. Flow cytometry and cell sorting

Cells were incubated with 0.1 µg antibody/10^6 cells: anti-CD3, anti-CD15, anti-CD33, anti-CD34, anti-CD38, anti-CD41, anti-CD61 (BD Biosciences), anti-BMPRIa, anti-BMPRIb (R&D Systems), anti-CD14, anti-CD19 (Beckman Coulter), anti-GPA (Invitrogen) at 4°C for 30 minutes. Conjugated isotype-matched controls were used to assess nonspecific fluorescence. Flow cytometry analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson) gated on viable cells. Cell sorting was performed using a concentration of 5-10x10^6 cells/ml on a FACSAria cell sorter (BD Biosciences) gated on viable cells. To determine the frequency of single-cell CFC formation, cells were directly sorted into U bottom 96-well ultra low attachment plates.

3. ELISA assay

The supernatant of bone marrow samples obtained from healthy donors and CML patients at diagnosis or during follow-up was harvested and cleared. Various dilutions of this processed supernatant were placed in 96-well plates (100 µl/well) to quantify proteins using the human BMP2-ELISA (Quantikine, R&D) or BMP4-ELISA (DuoSet, R&D) kits following the manufacturer's instructions. Measures were done in triplicate and repeated several times for the same experiment. The optical density at 450 nm was determined using a microplate reader (Dynex MRX).

4. Western blot analysis

Whole cell extracts were prepared by lysis of cells in 2X protein loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 5% b-mercaptoethanol, 0.01% bromophenolblue). Equal amounts of total protein were separated by sodium dodecyl sulfate polyacrylamid gel electrophoresis. Western blotting was carried out according to standard techniques. Antibodies were diluted in 5% non-fat milk in Tris-buffered saline, 0.1% Tween 20 (Sigma). The status of Twist-1, BMPRIb, Abl, Bcr-Abl and GAPDH proteins was analyzed by immunoblotting, using mouse monoclonal antibodies recognizing Twist-1 (abcam), BMPRIb (abcam), Abl (BD Pharmingen) and GAPDH (abcam) followed by incubation with an anti-mouse antibody conjugated with horseradish peroxidase (invitrogen) and Lumi-Light Plus chemiluminescent substrate (Roche). The films were scanned and quantification of the intensity of the bands was performed using ImageJ.

5. Transfection

TF1-GFP and TF1-GFP-BCR-ABL cell lines were transfected by electroporation with 1 µg of plasmid per 10^6 cells using Neon™ Transfection System (Invitrogen). The vectors pCAG-ires and pCAG-ires-BMPRIb were designed in our laboratory (details available upon request). Transfected cells were selected and maintained under Puromycin (1 µg/ml) (InvivoGen) treatment for 6 weeks before performing the experiments. Transfection efficiency was measured by flow cytometry for the expression of the cell surface BMPRIb protein.

6. Immunofluorescence staining

Cells were seeded at $5x10^5$ cells/mL and incubated for 24 hours before the experiment. In one hand, suspended cells were centrifuged onto glass slides by using a cytospin 4 (Thermo Scientific). In the other hand, suspended cells were allowed to adhere on glass cover slips coated with fibronectin (Sigma) in culture treated plates (BD Biosciences/Falcon) for 1 hour at 37°C, before removal of the non-adherent fraction. Cells were then fixed with 4% paraformaldehyde (PAF) for 15 minutes, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 minutes, and blocked with 0.2% gelatin (Sigma-Aldrich) for 30 minutes at room temperature. The filamentous actin (F-actin) was labeled with 1000-fold diluted phalloidin-rhodamin (Sigma-Aldrich) for 30 minutes at room temperature. Finally, cells were washed twice and and subsequently the nuclei were labeled with 2000-fold diluted DAPI (Sigma-Aldrich) in mounting medium (Sigma-Aldrich). Controls for autofluorescence were performed by incubating cells without phallodin-rhodamin. Fluorescence images were taken using a spectral confocal microscope TCS SP5 AOBS (Leica).

7. Immunohistochemistry

Immunohistochemistry was performed on bone marrow resin-embedded core biopsy specimens, after endogene peroxydase inhibition by H_2O_2, heat-induced antigen retrieval with DAKO buffer, pH6, antigen labeling overnight with anti BMP2 or anti-BMP4 antibodies (5 µg/ml), Envision lavelling (Dako), tyramide amplification (Perkin Elmer), followed by streptavidine-phosphatase alkaline (Invitrogen) and Liquid Permanent Red (Dako) revelation.

8. Ki67 staining

Ki67 staining was performed following the manufacturer instructions (BD Biosciences). Cells were fixed with 4% paraformaldehyde (PAF) for 15 minutes, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 minutes, and blocked with 0.2% gelatin (Sigma-Aldrich). Cells were then incubated for 30 minutes at room temperature with the antibody (Ki67-PE) and washed twice with PBS1X before analysis. Flow cytometry analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson) gated on viable cells.

9. Hoescht staining

Suspended hematopoietic cells were centrifuged onto glass slides by using a cytospin 4 (Thermo Scientific). Cells were then fixed with 4% paraformaldehyde (PAF) for 15 minutes, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 minutes, and blocked with 0.2% gelatin (Sigma-Aldrich) for 30 minutes at room temperature. Cells were washed twice with PBS1X and stained for 30 minutes with 1 µg/mL Hoescht 33258 (Life Technologies). Finally, cells were washed twice and mounted using mounting medium (Sigma-Aldrich). The percentage of dividing cells was then scored using an axiovert 25 microscope (Zeiss).

10. Cell-cycle analysis

Cells were fixed in 70% ethanol before being labeled with the staining solution (PBS 0.1% Triton X-100 (Sigma), 200 µg/ml DNAse-free RNAse A (Macherey-Nagel) and 20 µg/ml propidium iodide (Sigma)). Cell cycle analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson). Data was analyzed using the Watson model on FlowJo (Tree Star Inc).

III. Atomic Force Microscopy

1. Principle

Atomic force microscope (AFM) is a local probe technique, designed to measure interaction forces between a sharp tip and the surface of a sample. The tip is attached to a force-sensitive lever, which is fixed at the opposite end to a solid support that is called cantilever. Tip and sample are positioned relative to one another with nanometer precision by mounting either the solid support base of the lever or the sample support to a piezo-electric scanner (tip-scanning or sample-scanning microscopes, respectively). Hematopoietic cells were immobilized using fibronectin-coated cover slips or micro-fabricated wells (Figure 26A). An electro-mechanical step motor is also used for vertical course adjustments and initial approach of tip to sample. As the tip is brought toward the sample, it will eventually come into contact with the sample surface, and the lever will be deflected proportionally to the force experienced by the tip (at least within a certain limit of small deflections: the linear regime) (Figure 26B). The spring constant of the cantilever (0,01 to 0,1 N/m for biological applications) is calibrated with the thermal noise method. The microscope rests on a vibration-damping air table to minimize background signals as much as possible. Since the soft levers used for biological applications have resonance frequencies in the range of 1 to 20 kHz, they are particularly sensitive to acoustic vibrations. For this reason the AFM used here is enclosed in a custom built acoustic isolation box. The deflections of the lever are typically measured by optical beam deflection. For optical beam deflection, laser light is focused onto the back surface of the lever (opposite to the tip). The reflected laser light is directed onto a quadrant photo-diode. Voltage outputs are proportional to vertical or lateral deflections of the lever resulting from normal or tortional forces on the tip, respectively. Measurements can be performed in aqueous environments, lending itself as a useful tool for studying living cells in nearly physiological environments. AFM can also be combined to a variety of optical microscopy techniques such as inverted optical microscope or even fluorescent microscopy, further expanding its applicability. This allowed us to study the mechanical properties of single living cells.

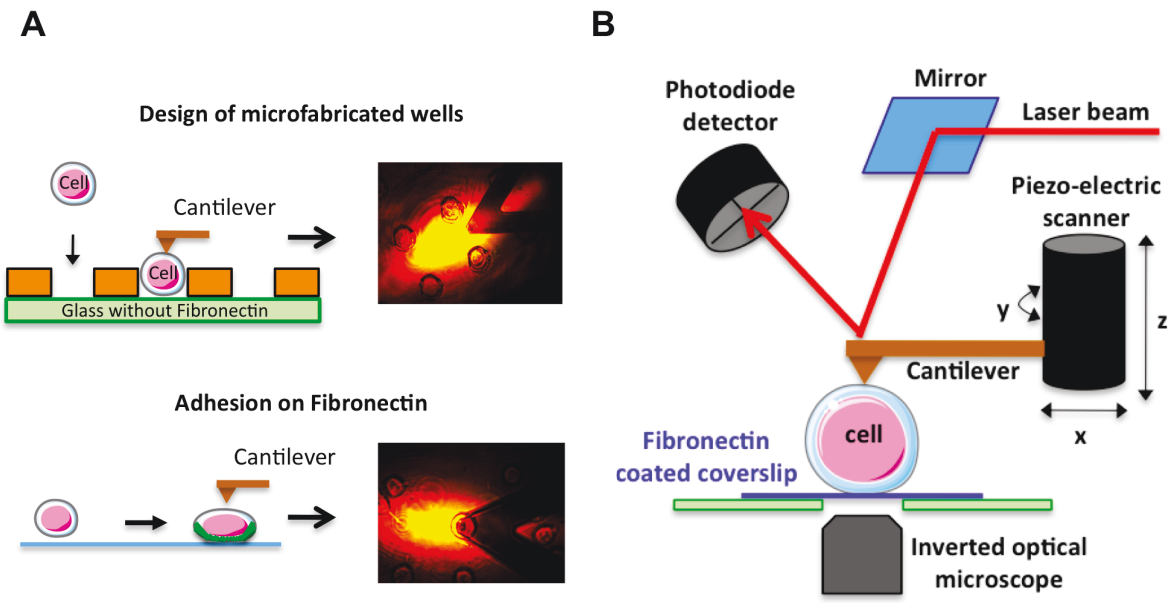

Figure 26 : AFM experimental setup. (A) Hematopoietic cells were immobilized using fibronectin-coated cover slips or micro-fabricated wells. (B) Single living cells were probed using an AFM cantilever controlled by a piezoelectric scanner and a laser tracking system. A sharp tip is moved back and forth towards the sample until a set point is reached and a force curve is generated.

2. AFM experiments

The AFM measurements were performed by means of an AFM/STM 5500 (Scientec) and a Nanoscope IIIa (JPK, Bruker), using an SNL-10 silicon-tip on nitride AFM cantilever (SNL-10; JPK, Bruker) having a spring constant of 0.06 N/m to indent the cells. For each experiment, the deflection sensitivity of the cantilever was calibrated on fused silica and the cantilever stiffness k was calibrated by the thermal noise method. In each case, the setpoint amplitudes and feedback control gains were chosen to minimize the error maps. Before force microscopy measurements were made, cells were seeded at $5x10^5$ cells/mL and incubated for 24 hours. For suspended cells measurements, cells were pipetted onto the wafer and allowed to settle into the micro-wells. For adherent cells measurements, suspended cells were allowed to adhere on glass cover slips coated with fibronectin (Sigma) in culture treated plates (BD Biosciences/Falcon) for 1 hour at 37°C, before removal of the non-adherent fraction. During the experiment, the glass coverslip was mounted on the AFM stage and the cells were kept in their culture medium at room temperature (24°C). Indentation was carried out at the center of each cell within 2 hours after cells were removed from the incubator at Z scan velocity 1 $\mu m.s^{-1}$. In all the experiments, images were taken to measure the size of each cell. Analysis was performed on data from series of 30 force curves per cell.

3. Modeling the force-indentation curves

Generalizing AFM force measurements to non-adherent cells was previously performed on myeloid cells, but these authors did not consider that these cells could change their mechanical response during their deformation, and they parameterized the force curves with a Hertzian model.[298] To capture changes in cell mechanical viscoelasticity during indentation, we used a strategy inspired from previous studies in material sciences for pyramidal indentation of viscoelastic solids. The Sneddon's variation of the Hertz model describes the evolution of the force F with the indentation I, for the case of a pyramidal (or conical) and non-adhesive cantilever tip:[461]

(Equation 1)

$$F(I = Z - Z_c) = \frac{\xi}{2} \tan\alpha \frac{E}{1-\nu^2} I^2$$

where ξ=1.4906 for a pyramidal shape tip, α is the half tip angle, Z_c is the contact position of the cantilever, E and v are the Young's modulus and Poisson ratio of the sample. This Hertz-Sneddon model assumes that the tested material is stationary, therefore it gives an estimation of the static Young's modulus E. When the sample visco-elasticity is not stationary (typical of an active material), differential operators must be used:[462]

(Equation 2)

$$F(t) = \frac{\xi \tan\alpha}{(1-\nu)} \int_0^t G(t-\tau) dI^2(\tau)$$

G(t) is the time-dependent shear modulus of the material and I(t) is the cantilever indentation inside the material. This shear modulus is defined as the ratio of the shear stress to the shear strain. For a constant cantilever displacement rate, $I(t)=v_0 t$ for $t>0$ and $I(t)=0$ for $t<0$, then the previous equation becomes:

(Equation 3)

$$F(t) = \frac{2\xi\, v_0^2 \tan\alpha}{(1-\nu)} \int_0^t G(t-\tau)\tau d\tau$$

Taking the second derivative of this equation, we get:

(Equation 4)

$$\frac{d^2F(I)}{dI^2} = \frac{2\xi \tan\alpha}{(1-\nu)}G(I)$$

G(I) can thus be computed directly from the force-displacement curves by taking their second order derivative (their curvature). When G is constant, equation 4 yields equation 1, by replacing G by the Young modulus E=2G(1+v). Given that the stress field is axially symmetric for a pyramidal indentation, the force-displacement curve is written as an integral equation (Equation 2) that straightforwardly leads to the temporal shear modulus G(t) via a double derivation in time (Equation 4). As described in the next paragraph, this double derivation will be performed using the wavelet transform.

4. Wavelet Transform analysis of force curves

The continuous wavelet transform is a mathematical technique introduced in signal analysis in the early 1980s.[463] Since then, it has been the subject of considerable theoretical developments and practical applications in a wide variety of fields.[464,465] We used wavelet transform to smooth force-indentation curves and to detect the contact point. In mathematical terms the wavelet transform of a signal f(x) reads:

(Equation 5)

$$W_\psi f(a,b) = \frac{1}{a}\int_{-\infty}^{\infty} f(x)\psi^*(\frac{x-b}{a})dx$$

A typical analyzing wavelet ψ(x), that is admissible (of null integral) is the second derivative of a Gaussian $g^{(0)}(x)=e^{-x2/2}$, also called the Mexican hat wavelet:

(Equation 6)

$$g^{(2)}(x) = -\frac{d^2}{dx^2}g^{(0)}(x) = e^{-x^2/2}(1-x^2)$$

Via two integrations by part, we then obtain:

(Equation 7)

$$W_{g^{(2)}} f(a,b) = a^2\frac{d^2}{dx^2}W_{g^{(0)}} f(a,b)$$

This last equation shows that the wavelet transform computed with $g^{(2)}$ at scale a is nothing but the second derivative of the function f smoothed by a dilated version $g^{(0)}(x/a)$ of the Gaussian function. Each force curve was analyzed separately by a home-made Matlab script using the second derivative of the Gaussian as analyzing wavelet to compute directly the local curvature of the force curves (Equation 7). This second derivative of the force curve is directly related to the shear modulus G (Equation 4). The optimum wavelet scale for damping experimental noise and capturing correctly the curvature variation during indentation was chosen equal to 400 nm (scientec system) or 200 nm (JPK system). We used an analyzing wavelet of width 60 nm to find the contact points. The JPK system allowed to measure more points per curve and to decrease experimental noise. Then the shear modulus was estimated using Equation 4.

IV. Cell confinement.

To allow the testing of multiple conditions in the same experiments, we designed modified standard polystyrene 24-well plates (BD Biosciences/Falcon, 15 mm diameter) in which PDMS pillars fixed on the plate lid held the micro-structured slides (Figure 27)[273,458–460]. When the lid was closed with a clamp, the slides were maintained on the cells with a moderate pressure, allowing precise confinement height. Soft PDMS cylinders (0.5 MPa young modulus measured), 2.5% longer than the well depth, were molded and fixed on the lid of the plate. A 14 mm diameter confinement glass slide bearing PDMS micro-structures (fabrication described in materials section) was placed on the top of each cylinder. Cells were seeded in the wells at $2x10^5$ cells/mL. For non-adherent cells experiments, 24-well plates were coated with fibronectin (Sigma) to allow the adhesion of hematopoietic cell for 1 hour at 37°C, before removal of the non-adherent fraction. The percentage of adherent cells was measured for each experiment. After 2 hours of incubation in fresh medium, the modified lid was positioned on the plate and gently clamped, thus confining more than 85% of the adherent cells.

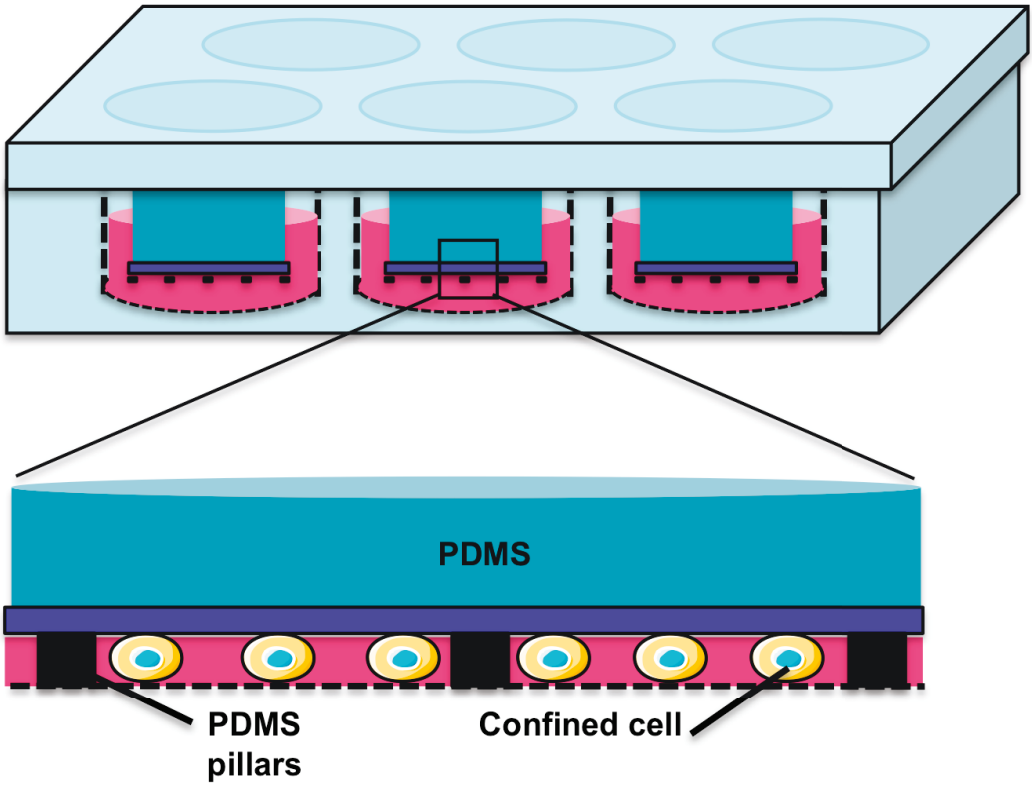

Figure 27 : Experimental setup for cell confinement. The lid of a multiwell plate is modified by introducing large PDMS pillars to hold the confinement slides. These pillars have the height of the plate, which can be slightly increased with a thin PDMS layer. When the multiwell plate containing adherent cells and culture medium is closed with the modified lid, the confinement slides are applied on the cells adhering to the bottom of the wells. Large pillars that are slightly higher than the well depth deform and apply pressure on the confinement slides.

V. Quantitative Phase Imaging

In the last decade, different teams have played a major role in disseminating the concepts of quantitative phase microscopy (QPM) for real time probing cellular structure and dynamics.[466–470] We built in our laboratory a variant of the device of Popescu and co-authors,[471] interposing an amplitude diffraction grating in the image plane of an inverted microscope to design simple and compact homemade interferometer, coupled to a high rate video camera.[472] The optical path length (OPL) measured by this microscope is defined as:

(Equation 8)

$$OPL = \int_0^H (n_C - n_0)dZ = \frac{\lambda\phi}{2\pi}$$

where n_C (respectively n_0) is the cell (respectively medium) refractive index, H is the height of the cell at position (X, Y), φ is the optical phase given by the interferometric measure, λ=532

nm is the optical wavelength. We use this microscope to compare the morphology of cells through their phase images, and to extract their OPL, which is directly related to the product of their thickness with their relative refractive index (compared to the buffer medium). We have here a fast and non-intrusive method for computing the cell refractive index and thickness.

VI. Statistical analysis.

Statistical significance was determined using the Wilcoxon-Mann-Whitney test in R-software version 2.9.2. Bivariate analysis of the variance was carried out using the ANOVA method. Significant p-values are indicated by an asterisk (*$p<0.05$; ** $p<0.005$; *** $p<0.0001$).

Results

Prmitive CML cell expansion relies on abnormal levels of BMPs provided by the niche and BMPRIb over-expression

I. Introduction

Chronic myelogenous leukemia (CML) likely arises from a stem cell (SC) transformation induced by the formation of the BCR-ABL oncogene. Without treatment, the disease evolves to an inexorable fatal blast crisis. Until recently, Imatinib has become the gold standard for chronic phase (CP) CML care. However, some discrete Philadelphia positive (Ph^+) leukemic stem cells (LSCs) may be insensitive to tyrosine kinase inhibitors (TKIs)[81,92,144] and therefore sustain detectable disease for many years. None of the therapeutic agents available to date seem to eradicate undifferentiated BCR-ABL^+ cells that serve as a reservoir for additional oncogenic events leading to disease progression[92,137,144,181] and require continued treatment.[473,474] Therefore, CML represents a unique model to study LSC biology and to elucidate some of the mechanisms of therapeutic resistance. Complete elimination of CML clones has rarely been achieved by TKI because of the development of a variety of cell-intrinsic and cell-extrinsic protective mechanisms. Extrinsic mechanisms are supported by the bone marrow (BM) microenvironment, the so-called “leukemic niche”. Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β superfamily and regulate hematopoietic SC fate both directly at various stages of SC differentiation[388,403,475] and indirectly through the control of their microenvironment.[50,399] These molecules are naturally produced by stromal cells, megakaryocytes and platelets[399]. In humans, BMP2/4/7 regulate proliferation, maintenance,[402] clonogenicity and repopulating activities of immature cell populations.[388,411] In the absence of erythropoietin, BMP2 induces cell commitment and differentiation towards erythropoiesis,[404] while BMP4 sustains SC maintenance and megakaryocytopoiesis.[399] BMP2/4 are inhibited by follistatin or FLRG binding which are soluble glycoproteins naturally expressed in the human BM microenvironment.[404,476] Follistatin and FLRG are involved in the regulation of immature hematopoietic cell adhesion,[412] thus contributing to the regulation of human hematopoiesis.[476] Survival of CML LSCs appears to be independent from the BCR-ABL oncogene[181,477] which strongly suggests the involvement of other signals or of the microenvironment.[195] Interestingly, the TGF-β family has been involved in the maintenance of CML LSCs.[478] BMP signaling is often altered in numerous cancers and involved in cancer SCs properties.[358] Here we asked if soluble BMP levels in the bone marrow of patients with active CML are altered and whether this is associated with molecular and/or cellular alterations in the responses of LSCs to BMP.

II. Results

1. Molecular alterations in BMP signaling elements in CML cells

To evaluate whether the endogenous BMP pathway is deregulated in CML, we compared the gene expression profiles of the main elements of the BMP pathway (Figure 28A) in normal cells and CP-CML cells at diagnosis prior to any treatment, using quantitative PCR (qPCR). We found that the expression of several components was deregulated in CML cells (Figure 28B). When compared to normal BM, *Smad1* ($p<0.0001$), *Smad4* ($p<0.0001$), *Smad6* ($p=0.0062$) and *Id2* ($p=0.0025$) were significantly down-regulated while *BMPRIb* ($p=0.0005$), *Smad5* ($p=0.0455$) and *Runx1* ($p=0.00175$) were significantly up-regulated. We then investigated if these changes could also affect immature $CD34^+$ CML cells. While *Smad1* ($p<0.0001$), *Smad8* ($p=0.001$), and the target genes *Id2* ($p<0.0001$), *Id3* ($p<0.0001$) and Runx2 ($p<0.0001$) were significantly down-regulated, *BMPRIb* ($p=0.017$) and *Smad6* ($p=0.03$) were up-regulated (Figure 28C). These results indicate that the BMP pathway is altered in immature $CD34^+$ CML cells at all levels of the pathway. We then performed a paired analysis between $CD34^+$ and $CD34^-$ cells purified from the same CP-CML samples at diagnosis to compare gene expression between immature and mature cells of the same patient and calculated a ratio for each individual RNA expression. Almost 60% of the BMP-signaling components tested appeared to be differentially expressed between the two compartments. One of the most conserved alterations in both the $CD34^+$ and CD34- cell populations, when compared to their healthy counterpart, was the high levels of *BMPRIa* and *BMPRIb* transcripts together with the very low expression of *Smad1*. Our results indicate that a major change occurs in the BMP signaling, including an over-expression of type I receptors (BMPRI), a down-regulation of Smad-signaling elements and a striking inversion of target gene expression. These data show that the BMP pathway is affected at all levels in CML.

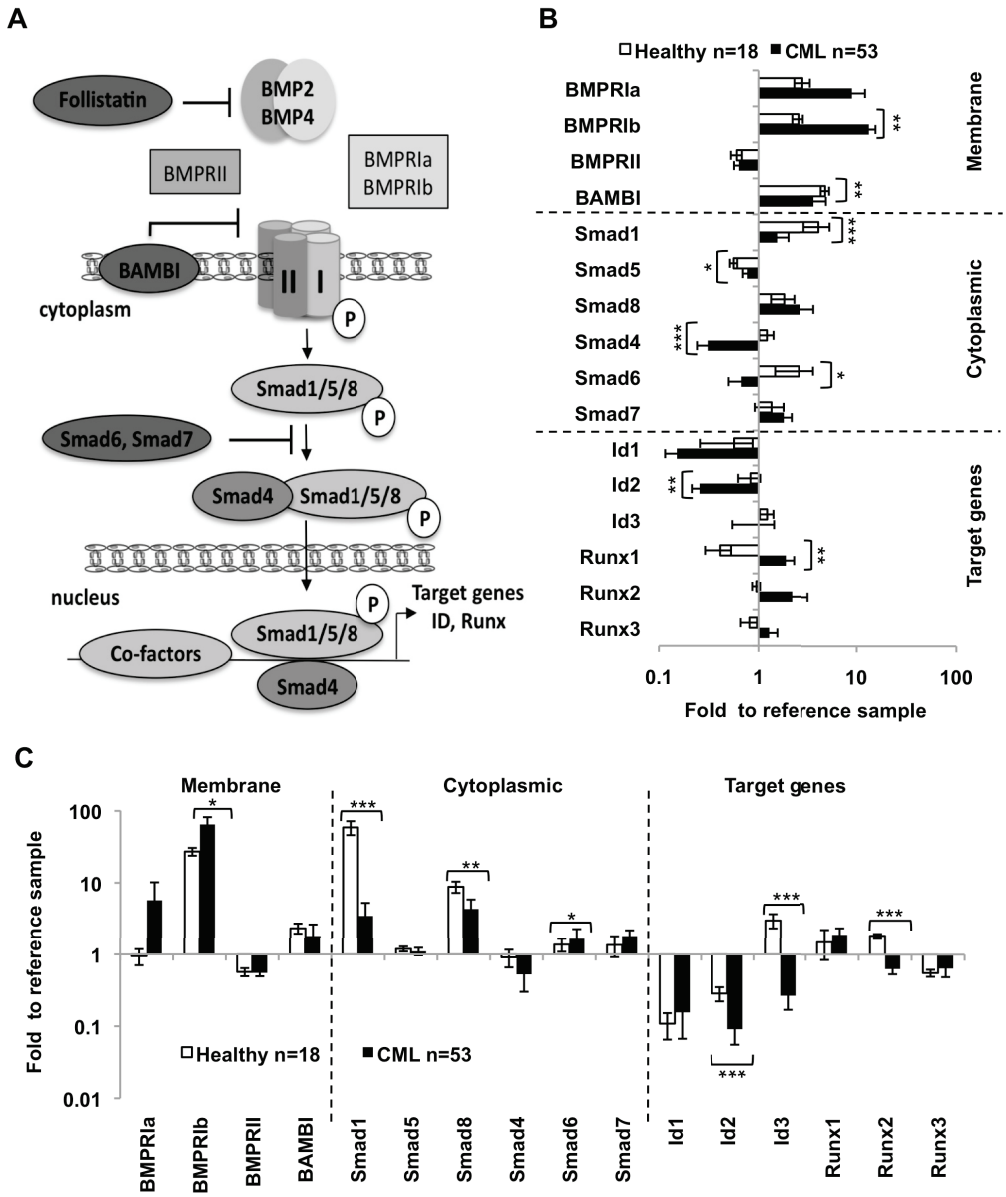

Figure 28 : Expression of BMP signaling elements in primary healthy and CML cells. (A) Representative scheme of the BMP signaling pathway that displays the main components of the signaling cascade including extracellular (soluble molecules), membrane, cytoplasm and nuclear located responding elements as well as some BMP target genes. Gene expression (by qPCR) of BMP signaling elements in healthy donor (open bars, n=18) and CP-CML samples at diagnosis (closed bars, n=53) for (B) the total number of mononuclear cells or (C) $CD34^+$ immuno-selected hematopoietic cells. Results are expressed as fold change versus the reference value obtained for each gene using the same $CD34^-$ healthy donor sample. *p<0.05; **p<0.005; ***p<0.0001 indicate differences between the gene expression levels in CML donor samples compared with healthy donor samples.

2. Deregulation of BMPRIb at the cell surface differentiates normal from immature leukemic cells

We analyzed gene expression levels in the following sorted cell subpopulations obtained from healthy donor BM samples (n=4) or CP-CML cells at diagnosis (n=3):

immature primitive cells ($C34^+$, $CD38^-$); immature progenitors ($CD34^+$, $CD38^+$); lineage restricted progenitors (C34-, CD38+); and cells differentiated as monocytes/granulocytes ($CD14^+$/$CD15^+$); myeloid ($CD33^+$); megakaryocytes ($CD61^+$/$CD41^+$); erythroid (Glycophorin A-GPA^+); T ($CD3^+$) and B ($CD19^+$) lymphocytes. We focused on BMPRI as mediators of the exogenous effects of BMP and encoded by one of the most significantly perturbed genes at diagnosis. The higher expression of the BMPRIa (Figure 29A) and of the BMPRIb (Figure 29B) was present in almost all of the CML subpopulations indicating that changes in the BMP pathway are a hallmark of leukemic subpopulations. We also analyzed their expression in cells from patients at diagnosis within the three established patient groups based on their Sokal score (low, intermediate or high) and at advanced stages (accelerated phase, blast crisis). *BMPRIb* expression progressively increased with disease phase conversely to that of *BMPRIa* (Figure 29C). However, while the increase of *Bmi1* was only observed in late stages of the disease according to previous data,[479] *BMPRIb* was highly up-regulated in CP-samples (>10-fold) as early as at diagnosis when compared with normal samples and again another 10-times more elevated during the advanced phases. These data suggest that the expression of BMPRIb is early modified during the course of CML.

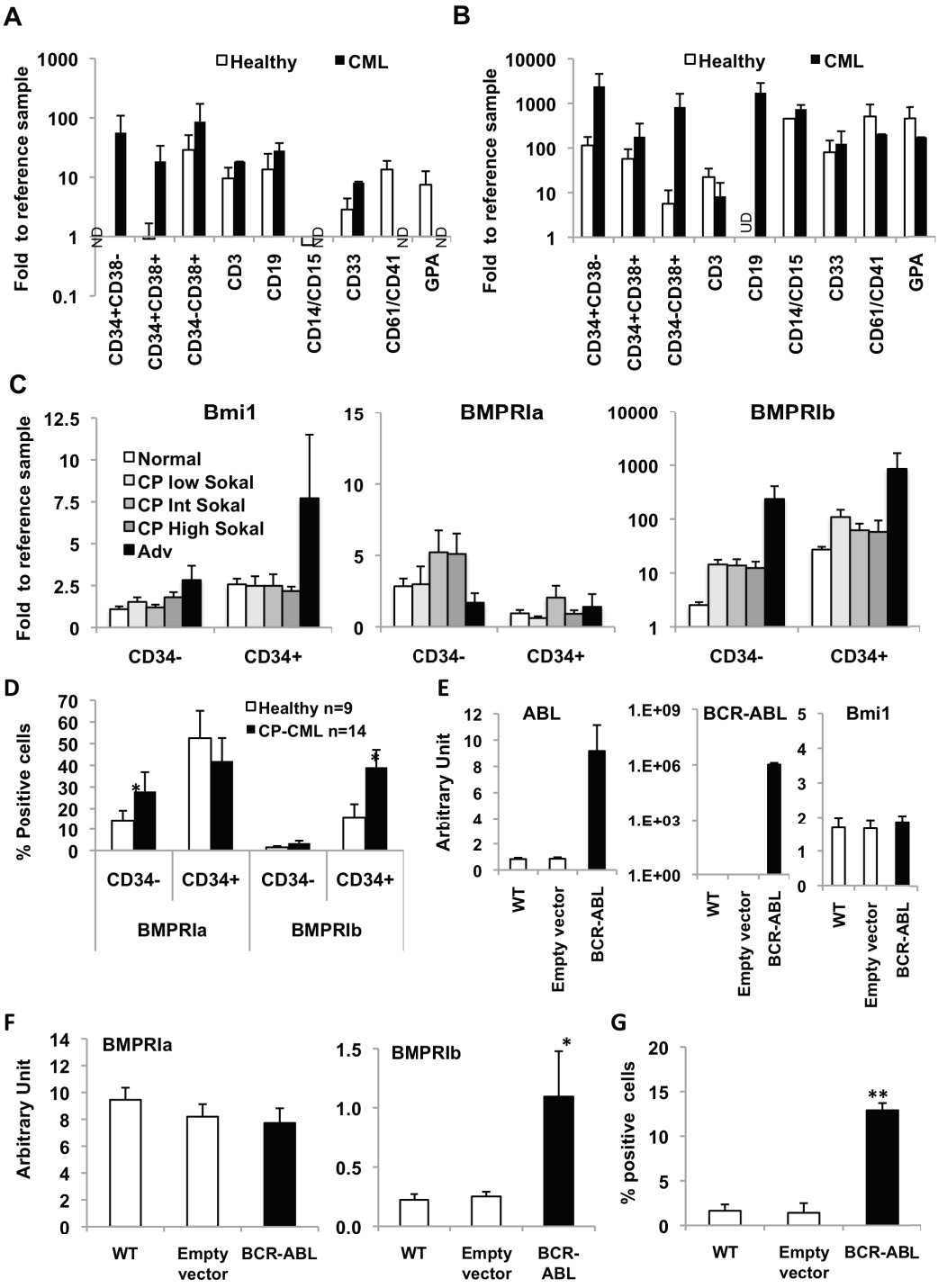

Figure 29 : BMPRIb is specifically deregulated in primary immature CD34$^+$ CML cells. Comparative expression of BMPRIa (A) and BMPRIb (B) genes in distinct sorted sub-populations obtained from healthy donor BM samples (n=4, open bars) and CP-CML patient samples at diagnosis (n=3, closed bars). The sub-populations represent: immature primitive cells (CD34$^+$, CD38$^-$), immature progenitors (CD34$^+$, CD38$^+$), lineage restricted progenitors (CD34$^-$, CD38$^+$), monocytes/granulocytes (CD14/CD15$^+$), mature myeloid cells (CD33$^+$), megakaryocytes (CD61/CD41$^+$), erythroid compartments (Glycophorin A-GPA$^+$), T (CD3$^+$) and B (CD19$^+$) lymphocytes. Results are expressed as fold change versus the reference value for each gene using the same healthy donor sample. *p<0.05; **p<0.005; ***p<0.0001 indicate differences between the gene expression levels in CML compared with healthy samples. (C) Comparative expression of Bmi1, BMPRIa and BMPRIb genes in CD34$^+$ and

$CD34^-$ cells obtained from healthy donors (n=14, open bars) and CML patients samples either in chronic phase (CP) at diagnosis (n=53, grey bars) or in advanced (Adv) phase at diagnosis (n=4, closed bars). Results from patient samples in chronic phase at diagnosis are sub-divided between low (<0.8), intermediate (0.8–1.2) or high (>1.2) Sokal score (different tone grey bars). (D) Flow cytometry analysis of BMPRIa and BMPRIb cell surface expression in $CD34^+$ and $CD34^-$ cells obtained from healthy donors (n=9, open bars) and CP-CML samples at diagnosis (n=14, closed bars) using a Facscalibur® cell analyzer (Becton Dickinson). Results are expressed as the % of receptor-expressing cells. *p<0.05 indicate differences between healthy and CML samples. Comparative expression of (E) ABL, BCR-ABL and Bmi1 genes or (F) BMPRIa and BMPRIb genes by qPCR in parental TF1 cells either transduced with an empty vector (non BCR-ABL, open bars) or with a vector containing BCR-ABL expressing sequence (closed bars). Results represent the mean value ± SEM of n=7 (E) or n=14 experiments (F). *p<0.05 indicates differences between parental TF1 cells transduced with an empty vector and BCR-ABL-transduced TF1 cells. (G) Flow cytometry analysis of BMPRIb receptor cell surface expression on either parental TF1 cells transduced with an empty vector (non BCR-ABL, open bars) or with a vector containing BCR-ABL expressing sequence (closed bars) and as indicated, performed on viable cells using a Facscalibur® (Becton Dickinson). Results represent the mean value ± SEM of n=8 experiments. (**p<0.005 indicates differences between parental TF1 cells transduced with an empty vector and BCR-ABL-transduced TF1 cells.

We analyzed BMPRI cell surface expression by flow cytometry. While the increased expression of BMPRIa reach statistical significance in mature $CD34^-$, BMPRIb was significantly over-expressed in immature $CD34^+$ CML cells (p=0.037 and p=0.04, respectively) (Figure 29D). We analyzed the impact of the introduction of BCR-ABL in the human cell line TF1, used as a model of immature $CD34^+$ cells that displays clongenic ability similar to human BM $CD34^+$ cells and able to differentiate into myeloid lineages.[480] When compared to a wild type (WT) cell line or when transduced with an empty vector, BCR-ABL-transduced TF1 cells (TF1-BCR-ABL) increased their transcriptional levels of BCR-ABL and ABL as expected (Figure 29E) but not levels of Bmi1 the deregulation of which more likely reflects a secondary event[479] (Figure 29C). While no difference was observed between transduced and un-transduced cells regarding the BMPRIa expression, the transcriptional level of BMPRIb increased in TF1-BCR-ABL cells (p=0.014, Figure 29F). This was confirmed at the protein level (p=0.0004, Figure 29G). These data indicate that the TF1-BCR-ABL model reproduces the deregulation observed in CP-CML patients regarding the BMPRI expression patterns that are directly driven by the BCR-ABL oncogene. Data in both primary cells and the TF1-model strongly suggest a change in the regulation of LSC by the BMP pathway.

3. Excess of both BMP2 and BMP4 within the tumoral environment contributes to early increases in BMPRIb expression in immature CML cells

We then investigated if soluble BMP involved in the regulation of normal HSC and progenitors[399,404,476] were present in the CML tumor environment. Using an ELISA assay, we measured the levels of BMP4 and BMP2 present in BM plasma obtained from healthy donors and CP-CML patients at diagnosis separated into two groups on the basis of their number of circulating platelets (elevated: >400G/l or normal: ≤ 400G/l) as a potential source of soluble BMPs[399] (Figure 30A). We observed a significantly higher level of BMP2 (p=0.0001) and of BMP4 (p=0.0008) in patients with normal platelet counts and a further increase in some patients with a deregulated platelet count; however, this increase was not significant. Unlike *BMP4, BMP2* transcripts are elevated in normal $CD34^+$ blood cells and low in $CD34^-$ cells from healthy donors (Figure 30B). In CML blood, lower levels of *BMP2/4* transcripts were detected in $CD34^+$ and $CD34^-$ cells isolated from the same samples. Conversely, primary leukemic stromal cells[481,482] displayed higher levels of B*MP4* transcripts and *BMP2* transcripts to a lesser extent (Figure 30C). We used BM biopsies to identify the in situ source of soluble BMP2 and BMP4 using antibody staining. We observed an increase in BMP2 staining in mature polynuclear (PN) cells and an increase in BMP4 staining in endothelial sinusoid cells (Figure 31D). We also detected higher levels of *BMP2* transcripts in CP-CML sorted BM-myeloid ($CD33^+$) and -megakaryocytic ($CD61^+$) cells, confirming the probable but partial contribution of the megakaryocytic cells to BMP2 levels together with PN cells (Figure 31A). Our data indicates that the leukemic microenvironment is the main source of soluble BMP2/4 involved in the maintenance and expansion of LSCs that do not produce these cytokines in an autocrine fashion in the chronic phase. Therefore, in the leukemic context, $CD34^+$ cells are exposed to higher concentrations of BMP2/4. We analyzed the effect of such a chronic exposure on the expression of the BMPRIb by treating TF1 cell lines with BMP2 or BMP4 for 4 weeks. Under these conditions, in the presence of the oncogene BCR-ABL, the levels of BMPRIb appeared to be maintained by BMP4 and significantly increased by BMP2 treatment (p=0.0268) while a slight but not significant effect was observed in non-transduced TF1 cells (Figure 30E).

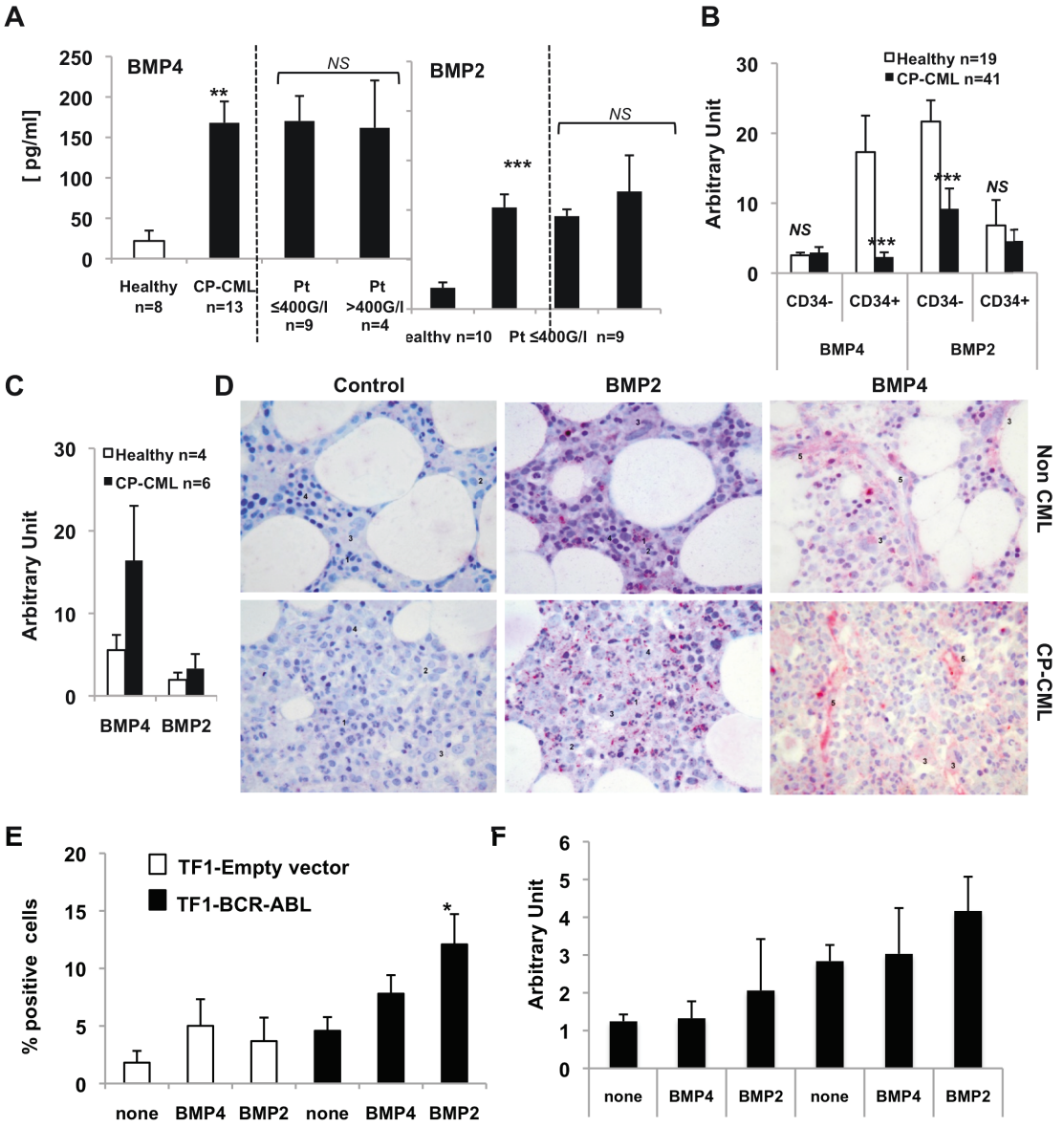

Figure 30 : High levels of BMP2 and BMP4 produced by the CML microenvironment contributes to BMPRIb induced expression. (A) ELISA quantification of BMP4 and BMP2 in BM plasma obtained from healthy donors and CP-CML samples at diagnosis. Data are categorized according to circulating platelet number measured for CML patients as either normal (≤400G/l) or abnormally elevated (>400G/l). Results expressed in pg/mL represent the mean value ± SEM of the indicated number of analyzed samples. *p<0.005 and ***p<0.0001 indicate differences between the levels of BMP2 and BMP4 in CP-CML samples at diagnosis compared with healthy samples. Comparative expression of BMP2 and BMP4 genes in (B) $CD34^+$ and $CD34^-$ cells obtained from healthy (n=19, open bars) and CML blood samples (n=41, closed bars), *p<0.0001 indicates differences between the expression of BMP2 and BMP4 genes in CML patient blood samples in CP at diagnosis compared with healthy samples; and in (C) stromal cells derived from 3 to 4 weeks of culture of un-manipulated BM samples from healthy (n=4, open bars) and at diagnosis CP-CML samples (n=6, closed bars). (D) In situ staining of BMP2 and BMP4 and their control antibody performed on sections of bone marrow biopsies from non-hematologic malignancy patients (n=2, upper panels) and CP-CML patients at diagnosis (n=3, lower panels). Pictures were captured on a DMR microscope using PL FLUOTAR objective (Leica) at a magnification of x40/1.00-0.50 oil. The following cells are indicated in the picture: (1) polynuclear neutrophils,

(2) immature granular neutrophils (promyelocytes/myelocytes), (3) megakaryocytes, (4) erythroblasts and (5) endothelial cells of the sinusoid. (E) Flow cytometry analysis of BMPRIb receptor cell surface expression on parental TF1 cells either transduced with an empty vector (non BCR-ABL, open bars) or transduced with a vector containing BCR-ABL expressing sequence (closed bars) after chronic exposure for 4 weeks to either BMP2 or BMP4 (50ng/mL). Results represent the mean value ± SEM of n=8 experiments. *p<0.05 indicates differences between parental TF1 cells transduced with an empty vector and BCR-ABL-transduced TF1 cells. (F) Selected CD34+ cells isolated from CP-CML samples were incubated in serum-free medium ($6x10^5$/mL) for 7 days in the presence of BMP4 or BMP2 (50ng/mL), with or without co-culture on a stroma composed of HS27A cells seeded at $2x10^4$ cells/mL 1 day before the experiment. Comparative expression of BMPRIb gene was then performed on CP-CML $CD34^+$ cells. Results represent the mean value ± SEM of n=3 experiments.

Similar results were obtained using primary $CD34^+$ CP-CML cells which displayed an amplified *BMPRIb* expression when co-cultured with the immortalized human BM-stroma cell line $HS27A^{483}$ that over-produces BMP2/4 (Figure 31B and Figure 30F). Together, our results show that immature leukemic cells not only display intrinsic alterations due to the BCR-ABL oncogene but also increased and sustained high levels of BMPRIb expression regulated by their niche.

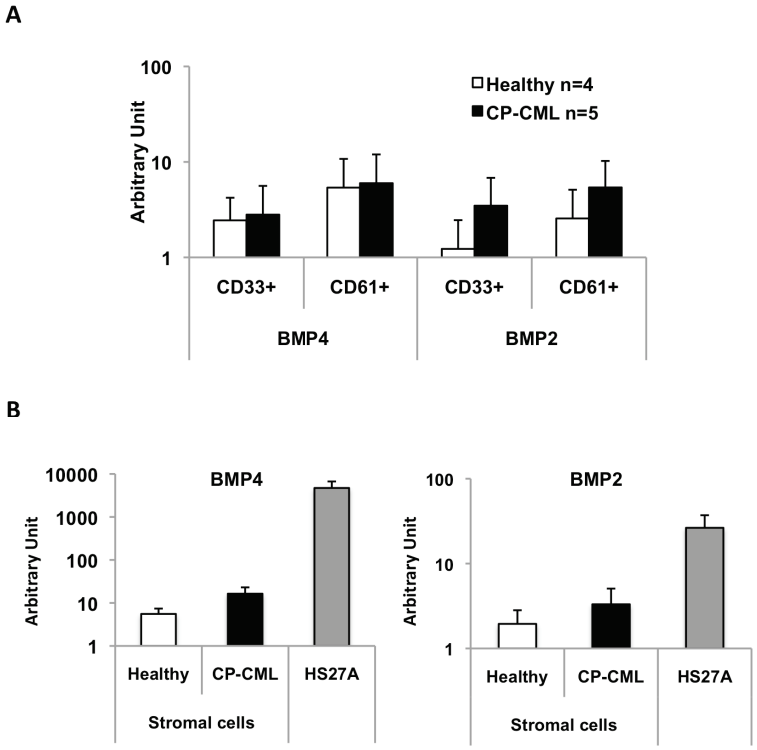

Figure 31 : Expression of BMP2 and BMP4 in distinct sub-populations. (A) Comparative expression of BMP4 and BMP2 genes in distinct sorted subpopulations obtained from healthy donor BM samples (n=4, open bars) and CP-CML patient BM samples at diagnosis (n=5, closed bars). The subpopulations represent: mature myeloid cells ($CD33^+$) and megakaryocytes ($CD61^+$). (B) Expression of BMP4 and BMP2 genes in HS27A cell line (n=6) compared with stromal cells from healthy donors (n=4) and CP-CML patients (n=6). Results are expressed as a fold change versus the same reference sample.

4. BMP2 and BMP4 expand immature CD34+ cells from CP-CML patients

Deregulations in the BMP pathway in immature leukemic cells together with a significant increase in available soluble BMP within the leukemic environment predict a perturbed response of LSC to BMP. To investigate this hypothesis, we compared the biological response of $CD34^+$ immature cells isolated from healthy BM donors or CP-CML patients at diagnosis to soluble BMP4 and BMP2. $CD34^+$ cells were incubated for 7 days in serum-free medium supplemented with BMP2 or BMP4 (50ng/mL) and analyzed for cell viability, proliferation and CD34 cell surface expression. We observed no differences in cell viability (Figure 32A) and proliferation (Figure 32B) compared with untreated cells (none), which suggests a lack of toxic effects of BMP2 or BMP4 in primitive cells. The number of CD34-expressing cells increased in the CML samples in the presence of BMP4 ($p=0.03$) and BMP2 ($p=0.026$) but not in healthy donors (Figure 32C). This increase in $CD34^+$ cells suggests either higher survival rates/maintenance or the amplification of LSC, as shown for BMP4 in normal HSC.[399,411] We then examined the quantities of stem and progenitor cells within the BMP-treated CD34+ cells using LTC-IC and CFC assays. BMP2 had no effect but LTC-IC numbers increased with BMP4 by 5.6-fold ($p=0.06$) in healthy cells and similarly by 4.7-fold in leukemic cells (Figure 32D). However, 58% (7/12) of healthy $CD34^+$ cells were expanded for their LTC-IC content by BMP4 and this frequency decreased to 34% (3/9) in CP-CML samples. Surprisingly, while less CML $CD34^+$ cells responded to BMP4, when they did, it was more pronounced as indicated by the large number of LTC-IC colonies we detected (data not shown). The progenitor compartment (CFC) was increased by both BMP4 and BMP2 treatment in healthy (1.6-fold and 1.5-fold $p=0.024$, respectively) and leukemic samples (2.5-fold, $p=0.033$ and 2.4-fold $p=0.022$ respectively) (Figure 32E). To establish whether the maintenance or amplification of CFC or LTC-IC by BMP affect remaining residual healthy or leukemic cells, we harvested individual colonies and analyzed them by qPCR for their expression of the normal Abelson (ABL) gene and the BCR-ABL oncogene. Colonies derived from healthy samples expressed ABL, but not BCR-ABL (data not shown). In the presence of BMP2 (5/5) and BMP4 (21/22), colonies detected in LTC-IC assays from patient samples were truly leukemic as over 95% of them expressed BCR-ABL (Figure 32F). All single CFC colonies (100%) derived from treated $CD34^+$ cells expressed BCR-ABL. These results indicate that BMP2/4 specifically affect the leukemic primitive cell compartment. In summary, these results indicate that changes in the expression of multiple BMP signaling factors correlate with a deregulated and amplified biological response in leukemic $CD34^+$ cells after exposure to soluble BMP.

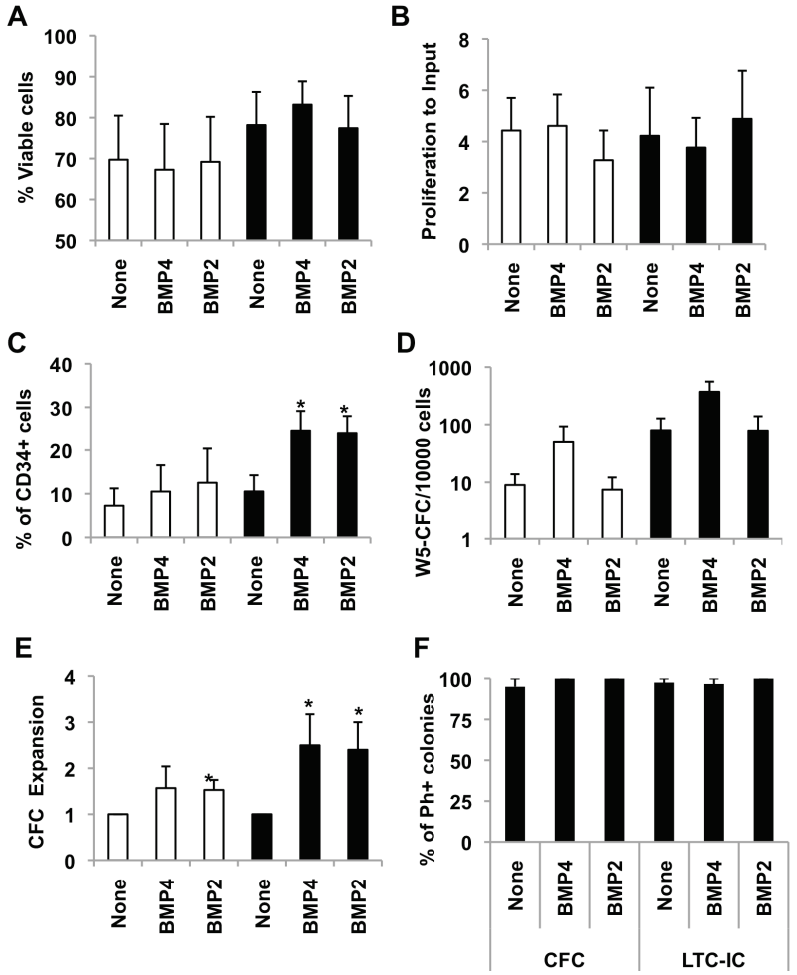

Figure 32 : Effects of BMP2 and BMP4 treatments on leukemic progenitor amplification. Selected $CD34^+$ cells isolated from healthy donors (open bars) or CP-CML (closed bars) samples were incubated in serum-free medium ($6x10^5$/mL) for 7 days in the presence of BMP2 or BMP4 (50ng/mL). *$p<0.05$ indicate differences between healthy and CML samples. Cell viability (A) and proliferation (B) were evaluated using trypan blue staining and cell counting. Results are expressed as the % of viable cells or the proliferation ratio that is the ratio of cell counts to the number of input cells. The mean ± SEM of n=10 experiments is presented. (C) Phenotypic analysis of CD34, the hematopoietic progenitor marker, was performed using flow cytometry on a Facscalibur® cell analyzer (Becton Dickinson) and gated for viable cells. Results are presented as the mean ± SEM of n=5 experiments for healthy donors and n=8 experiments for CML patients. (D) Stem cell content of treated-cells was analyzed by the LTC-IC co-culture assay and results are presented as the mean value ± SEM for of n=12 or n=9 experiments for healthy donors and CML patients, respectively. Results are presented as the total LTC-IC derived colonies after 5 weeks of co-culture per $1x10^4$ seeded cells. (E) The progenitor content of treated-cells was analyzed by the clonogenic CFC assay. Results are expressed as the ratio of treated to untreated cells and represent the mean value ± SEM of n=12 or n=19 experiments for healthy donors and CML patients, respectively. (F) The number of leukemic colonies in either CFC- or LTC-IC-derived colonies was assessed by picking individual colonies to quantify expression of ABL, BCR-ABL and the reference genes BGUS and TBP by qPCR. Leukemic positive colonies were determined by simultaneous detection of all genes while normal negative colonies were stated as ABL positive but BCR-ABL negative. In all cases, internal gene controls were positive, otherwise those samples would have been removed. Results are expressed as the % of positive BCR-ABL colonies out of the total assayed colonies of n=3 experiments for healthy donors and CML patients.

5. BMP2 and BMP4 induce the specific amplification of committed myeloid progenitors

To confirm the effect of BMP2/4 on leukemic $CD34^+$ progenitors, we analyzed their activities on the different categories of clonogenic cells (Figure 33).

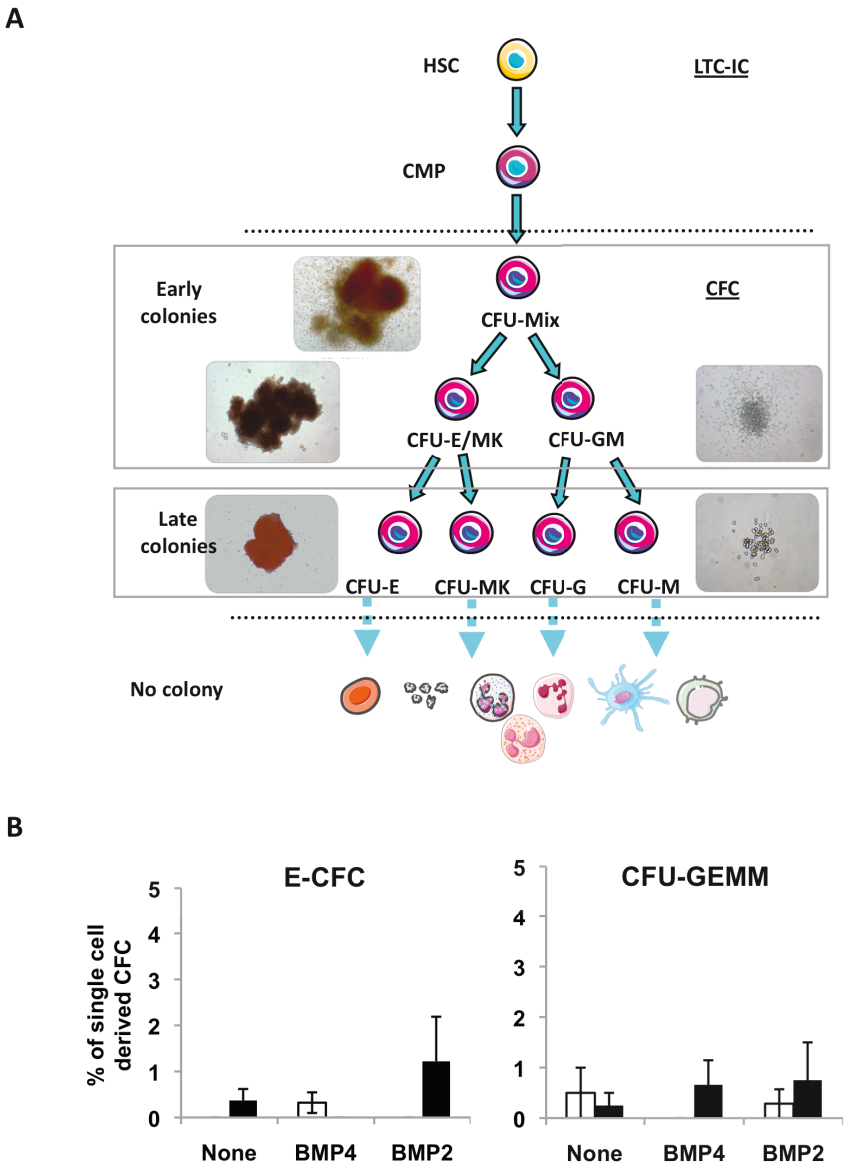

Figure 33 : Effect of the BMPs on the clonogenic potential of primary single cells. (A) Illustration of the different functional assays output throughout hematopoiesis differentiation scheme. The content of treated cells in distinct hematopoietic progenitor categories was determined using the clonogenic CFC assay. We scored them as early erythroid (over three clusters) and myeloid (over $1x10^3$ cells/colony) as well as late erythroid (one to two clusters) and myeloid (below $1x10^3$ cells/colony). (B) Sorted $CD34^+$, $CD38^-$ stem cells from healthy and CML samples were plated at one cell/well and were incubated in serum-free medium for 7 days in the presence of 50ng/mL BMP2 or BMP4. After 7 days, methylcellulose was added to the wells and incubated for 2 weeks prior to scoring of single cell derived CFC colonies. CFC categories from data obtained in each condition as erythroid (E-CFC) and mixed colonies (CFU-GEMM) are presented here. Values of colony subtypes are expressed as a percentage of the total number of colonies obtained from five experiments for normal and CML.

The most significant effects were observed in the granulo-monocytic compartment with a 9.6-fold increase in late leukemic CFU-GM induced by BMP4 treatment (p=0.045). BMP2-exposure led to a 13-fold increase in early granulo-monocytic progenitors (p=0.05, Figure 34A). No difference was observed in the number of erythroid or (CFU-GEMM) mixed colonies (data not shown). These data strongly support the notion that BMP2/4 are involved in the amplification of leukemic myeloid progenitors. We compared the effects of BMP2/4 in the same experiments on $CD34^+$ CML cells obtained from 18 patients. The absolute number of colonies, obtained in each condition from the same initial number of purified $CD34^+$ CML cells, was used to normalize the number of colonies obtained for the erythroid and granulo-monocytic categories and values were plotted as the percentage of progenitor subtype. Compared to untreated CML cells (54%), those treated with BMP4 or BMP2 amplified more granulomonocytic progenitors (61% and 65%, respectively) at the expense of erythroid progenitors (Figure 34B). The balance between granulo-monocytic and erythroid colonies thus significantly shifted towards a more granulo-monocytic phenotype with either BMP2 (p=0.01) or BMP4 (p=0.02) in a leukemic context, but remained unchanged for healthy cells. To evaluate if BMP2/4 induced LSC commitment towards the myeloid lineage, we sorted $CD34^+$, $CD38^-$ cells from CP-CML patients at diagnosis (n=5) and from healthy donors (n=5). The cells were plated at one cell per well in serum free conditions with or without BMP2 or BMP4. After 7 days, methylcellulose was added to the wells and single cell derived colonies were scored 2 weeks later. Consistent with the bulk culture results, less than 2% of normal single cells gave rise to colonies (Figure 34C) while the CML samples exhibited a strong increase in cloning efficiency (10–16%). Only the leukemic $CD34^+$, $CD38^-$ cells responded to BMP2/4, with a slight increase in total and CFU-GM colonies by BMP4 and no effect in the presence of BMP2. These data suggest that only BMP4 has a very weak effect on single cell proliferation and the clonogenic ability of the LSC while BMP2 is likely to contribute to the expansion of more differentiated myeloid progenitors.

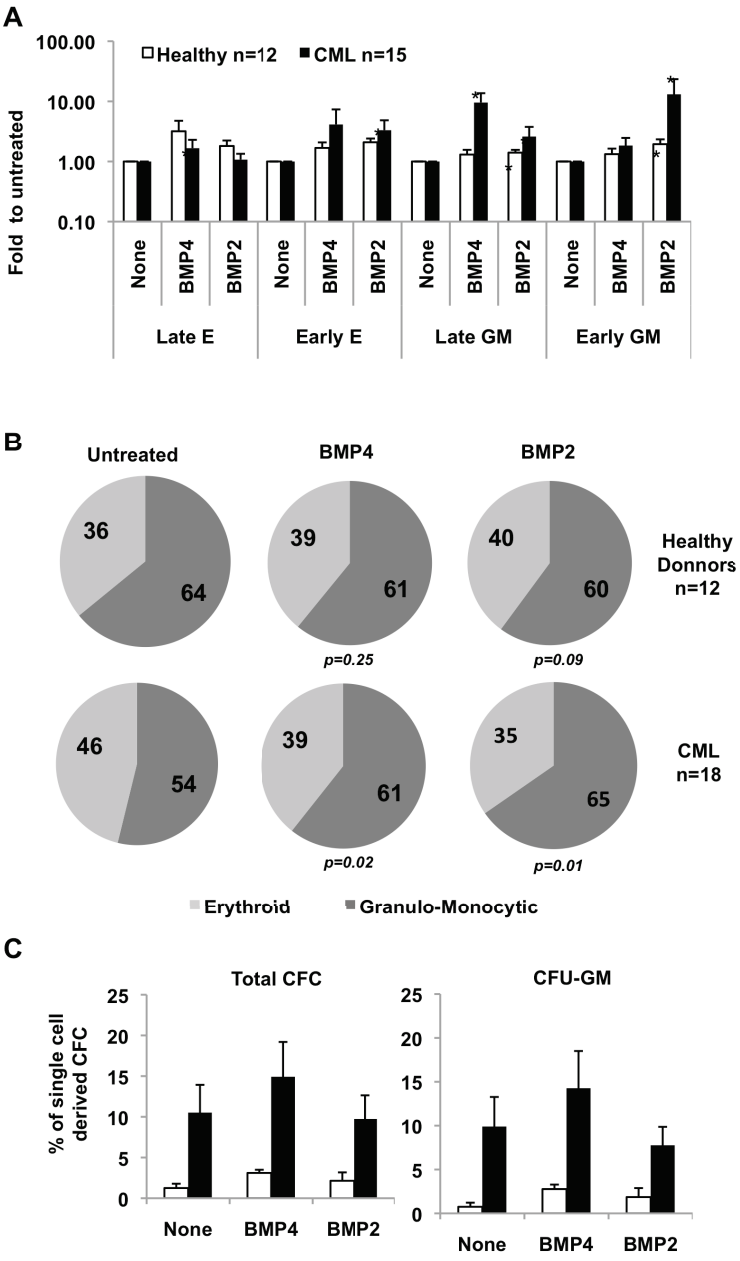

Figure 34 : BMP2 and BMP4 amplify leukemic myeloid progenitors. Selected $CD34^+$ cells ($6x10^5$/mL) were incubated in serum-free medium, for 7 days in the presence of either BMP2 or BMP4 (50ng/mL). The progenitor content of the treated-cells was analyzed using the CFC assay. (A) We scored them as indicated as early or late erythroid-E and granulo-monocytic-GM. Results represent the mean value ± SEM of n=12 or n=15 experiments for healthy donors and CML patients respectively and are expressed as ratio of treated to untreated cells (none). (B) CFC values obtained from $CD34^+$ cells treated in the same experiments with BMP2 or BMP4 and isolated from n=12 or n=18 different healthy or CP-CML patients at diagnosis respectively are expressed as a % of the total number of colonies obtained and presented as pie charts. $p<0.05$ indicate differences between treated and untreated cells. (C) Sorted $CD34^+$, $CD38^-$ cells from healthy and CML samples were plated at one cell per well in serum-free medium in the presence of 50ng/mL of BMP2 or BMP4. After 7 days, methylcellulose was added to the wells and single cell-derived colonies were scored 2 weeks later. We scored all colonies (total CFC) as well as for CFU-GM content. Results are expressed as % of wells that give rise to colonies and represent the mean value ± SEM of n=5 experiments for healthy donors and CML patients.

6. BMPRIb drives primitive leukemic myeloid progenitor amplification

To characterize the direct involvement of the BMP pathway in the amplification of leukemic myeloid progenitors we analyzed the effect of BMP2/4 treatments on the CFC and LTC-IC output of primary $CD34^+$ cells. Cells were isolated from healthy donors or CP-CML patients but divided into two groups on the basis of the level of *BMPRIb* gene expression. The *BMPRIb-low* group expressed transcript levels similar to normal samples (<10 arbitrary units) contrarily to the *BMPRIb-high* group that had transcript levels increased by 10-fold. In contrast to the *BMPRIb-low* patients, the *BMPRIb-high* group of samples strongly responded to BMP4 or BMP2 exposure, as measured by a major increase in LTCIC (107-fold; p=0.0018 and 15-fold; p=0.003, respectively) (Figure 35A) and CFC (4-fold; p=0.05 and 4-fold; p=0.008, respectively) (Figure 35B). These data suggest that while BMP4 has a very weak effect and BMP2 has no effect at all on cell fate decisions, in the overall CP-CML analysis of LSC, BMP2/4 both appear to induce a very strong expansion of LSC over-expressing BMPRIb that correlates to CFC amplification. Using the TF1-BCR-ABL model, we reproduced the CFC expansion following BMP4 or BMP2 chronic exposure to a similar extent as measured in primary cells with a 3.5-fold and 6-fold CFC increase in the presence of BMP4 or BMP2, respectively (Figure 35C) and no effect with control cells (TF1-Empty vector). The introduction of BMPRIb was by itself sufficient to increase the CFC number by 2.1-fold (p=0.003) while BCR-ABL alone led to only a 1.5-fold increase (p=0.05) (Figure 35D). Introducing BMPRIb into TF1-BA cells did not further expand CFCs. Lastly, we treated primary $CD34^+$ cells taken at diagnosis from CP-CML patients from BMPRIb-low or -high groups with soluble forms of the BMPRIa (sBMPRIa) or the BMPRIb (sBMPRIb) using previously determined optimal conditions[399] to out compete BMP binding to endogenous membrane-bound receptors. The BMP2/4-mediated CFC-increase was reduced in the presence of sBMPRIb and inversely increased when incubated with sBMPRIa (Figure 35E). Similarly, a significant (p=0.042) inhibition by of BMP2-driven CFC expansion was observed (Figure 35F) by treating TF1 cells transduced or not with BCRABL for 48 hours with sBMPRIb. These data confirm that changes in BMPRIb expression are associated with an alteration of the BMP2/4 response involved in the amplification of the leukemic myeloid stem and progenitor compartment in CP-CML patients.

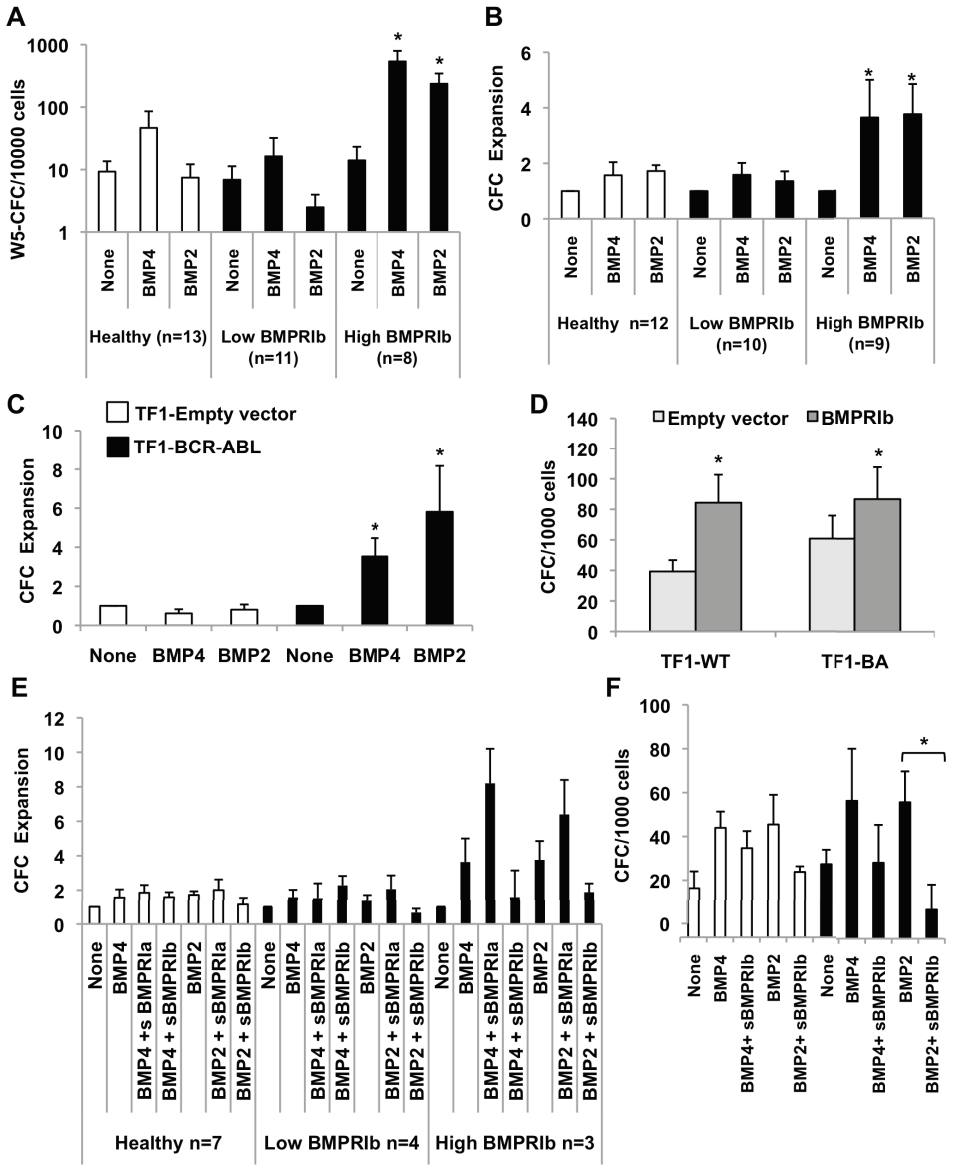

Figure 35 : BMPRIb receptor mediates BMP2 and BMP4 effects on LSC and myeloid progenitors. Following qPCR analysis of BMPRIb expression, CP-CML samples were divided as low and high BMPRIb-expressing CML samples. $CD34^+$ cells were then isolated from healthy donors (open bars) or both groups of CP-CML samples (closed bars) and incubated at $6x10^5$/mL in serum-free medium, for 7 days in the presence of BMP2 or BMP4 (50ng/mL). (A) The stem cell content of the treated cells was analyzed using the LTC-IC co-cultured assay and results represent the mean value ± SEM of the indicated number of samples. Results are presented as the total LTC-IC derived week 5 colonies per $1x10^4$ seeded cells. (B) The progenitor content of treated cells was analyzed using the CFC assay. Results are expressed as ratio of treated to untreated cells and represent the mean value ± SEM of the indicated number of samples. (C) BCR-ABL- (closed bars) or empty vector-transduced (open bars) TF1 cells were continuously treated for 4 weeks by 50 ng/mL BMP2 or BMP4 then assayed by CFC assay for their progenitor content. Results are expressed as the ratio of treated to untreated cells and represent the mean value ± SEM of n=5 experiments. (D) Parental TF1 cells (TF1-WT) or TF1-BCRABL (TF1-BA) cells were transfected with a control empty (grey bars) or a BMPRIb-encoding (dark grey bars) vector. The effect on CFC output was analyzed using the CFC assay. Results are expressed as the total CFC colonies per $1x10^3$ seeded cells and represent the mean value ± SEM of n=7 or n=3 experiments for TF1-WT and TF1-BCR-ABL, respectively. (E) The progenitor content of

$CD34^+$ cells isolated from healthy donors (open bars) or CP-CML (closed bars) samples. The cells were divided as low- and high-BMPRIb-expressing CML-samples and were treated for 7 days in serum-free medium by BMP2 or BMP4 (50 ng/mL) in the presence or absence of soluble BMPRIa or BMPRIb receptor (4 µg/mL). Results are expressed as ratio of treated to untreated cells and represent the mean value ± SEM of the indicated number of experiments. (F) The progenitor content of parental TF1 cells (TF1-WT, open bars) or TF1-BCR-ABL cells (closed bars) was analyzed by CFC assay following 48 hours of treatment by BMP2 or BMP4 (50ng/mL) with or without soluble BMPRIb receptor (4 µg/mL). Results are expressed as the total CFC colonies per $1x10^3$ seeded cells and represent the mean value ± SEM of n=5 experiments. *$p<0.05$ indicates differences between parental TF1 cells transduced with an empty vector and BCR-ABL-transduced TF1 cells.

III. Discussion

In various cancers including leukemia, the SC niche is often deregulated without being transformed per se[85,484] but its contribution to the maintenance, survival and resistance of LSC has only started to be deciphered. Here, we report a significant increase in the availability of soluble BMP2 and BMP4 in the BM of CP-CML patients at diagnosis, combined with an over-sensitivity of LSC to these molecules. In line with a recent in vivo study that demonstrated that CML-associated changes in the microenvironment confer a selective growth advantage to LSC,[485] we revealed for the first time the crucial role of alterations in the BMP pathway during the early stages of CML. By analyzing more than 70 samples from CP-CML patients at diagnosis, we demonstrated that the expression of a multitude of elements in the BMP pathway are altered in almost all CML subpopulations; including the stem cell/progenitor compartment, indicating a pathologic deregulation of this pathway while not reflecting the prevalence of a specific subpopulation. Some changes become more striking during the course of disease progression, such as BMPRIb over-expression. Our results, together with the fact that we restricted our analysis to samples obtained from patients that only display the t(9;22) translocation without additional clonal abnormalities, suggest that the BMP signaling alterations represent an early event in the transformation process. We demonstrated that alterations in the BMP pathway do not prevent leukemic cells from responding to exogenous BMP2/4. These data were confirmed using a model of BCR-ABL-positive immature $CD34^+$ cells reproducing the main features observed in primary immature CP-CML cells. Using this model, we further showed that over-expression of BCR-ABL increased BMPRIb surface expression. The erythroid-myeloid progenitor balance changed in favor of the granulo-monocytic lineage when treating CML-$CD34^+$ cells with BMP2/4. Also, BMP4-expanded LTC-IC were all genotypically Ph+, suggesting that the biological response of $CD34^+$ cells to BMP2/4 is specifically altered in CML. It then suggests that BMP4-mediated amplification and maintenance of LSC and BMP2-dependent expansion of myeloid progenitors are related to a hypersensitivity to exogenous BMP2/4 signaling mediated by

BMPRIb over-expression. This would further explain how LSC, in contrast to normal HSC, respond to soluble BMPs, thus contributing to the vast increase in the myeloid compartment observed in CML. In summary, we have demonstrated the existence of molecular and functional alterations in the BMP pathway in CML cells, as well as alterations in the quantities of soluble BMPs present in the tumor niche itself.[486] These alterations are involved in the chronic phase of the disease through their role in the survival of LSCs as well as in the expansion of myeloid progenitors (Figure 36). The analysis of the BMP pathway may therefore represent an interesting prognostic tool, allowing the design of drugs directly targeting the reservoir of LSC.

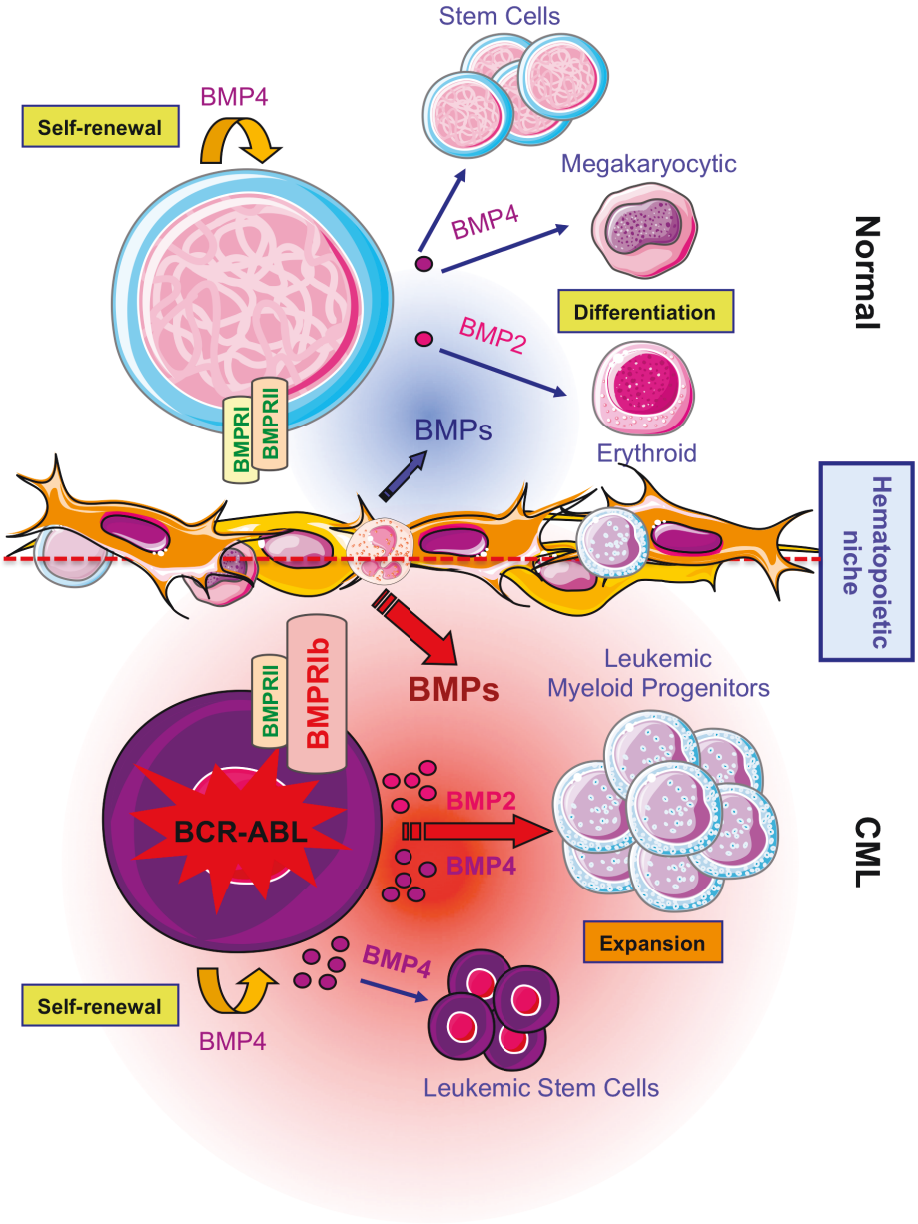

Figure 36 : Proposed model for BMP pathway alteration effects on CML LSC survival and myeloid progenitor expansion.

Role of BMP pathway alterations and soluble BMP production in LSC resistance to TKI

I. Introduction

One of the main causes of treatment failure in cancers is the development of drug resistance by cancer cells.[85] The persistence of cancer stem cells (CSC) might explain cancer relapses as they could allow reactivation of cancer cells proliferation following therapy, leading to disease persistence and ultimately to patients' death.[85] Clinically, it is crucial to develop therapeutic strategies able to target resistant CSC in order to cure the patients. In this context, Chronic Myelogenous Leukemia (CML) is the reference model of a true hematopoietic stem cell (HSC) alteration by a t(9,22) chromosomal translocation leading to the formation of the BCR-ABL fusion protein,[487] with high tyrosine kinase activity and directly responsible of the leukemic transformation.[92,105] Despite the success of targeted therapies against BCR-ABL with the development of tyrosine kinase inhibitors (TKIs),[131] a residual disease is still detected in many CML patients, probably due to the persistence in vivo of CML leukemic stem cells (LSCs).[81,92,144] None of the therapeutic agents available to date seem to eradicate these undifferentiated BCR-ABL$^+$ cells that may serve as a reservoir for additional oncogenic events.[92,144,181] In some cases, resistance to TKIs treatment appears, involving different mechanisms with 30% of unknown origin that could be due to LSC survival.[488] LSC are controlled by a variety of biochemical and biomechanical signals from the leukemic niche.[489] Thus, deregulation of these signals could contribute to LSC emergence and resistance. We demonstrated for the first time that alterations of intracellular BMP signaling pathway in CP-CML primary samples corrupt and amplify the response to exogenous BMP2 and BMP4, which are abnormally abundant in the tumor microenvironment.[486] Considering the crucial role of BMP pathway alterations in the maintenance of CP-CML LSC, these alterations could also influence LSCs resistance upon TKIs treatment. The importance of the BMP pathway in CML LSC is also illustrated by a recent discovery demonstrating that Twist-1, a bHLH embryonic transcription factor often regulated by BMPs,[446,447,490] constitutes a new predictive factor of TKI response in CML patients.[445] Interestingly, a reciprocal regulation between Twist-1 and BMP pathway elements has been demonstrated,[448,449] depending on the cellular context. Here we asked whether alterations of soluble BMP levels in the bone marrow of CML patients and/or alterations of BMP signaling pathway in CML LSCs could be involved in resistance to TKIs.

II. Results

1. Increase of BMP2 and BMP4 production with the emergence of resistance in tumor microenvironment

We first investigated if soluble BMP involved in the amplification of immature CP-CML cells were present in the CML tumor environment under TKIs treatment. Using an ELISA assay, we measured the levels of BMP4 and BMP2 present in BM plasma obtained from healthy donors, CP-CML patients at diagnosis, CML patients in complete cytogenetic remission (CCyR) and CML patients resistant to TKIs treatment. We previously observed an increase of BMP2 and BMP4 production in the CP-CML bone marrow environment.[486] Interestingly, we detected lower levels of BMP2 and BMP4 in patients that attained CCyR under TKIs treatment compared to CP-CML patients at diagnosis (Figures 37A and B). However, BMPs levels remained slightly higher compared to those observed in healthy donors bone marrow environment. In addition, the results observed for CML patients who developed a resistance to TKIs treatment are even more striking. Indeed, we observed an increase of both BMP2 and BMP4 levels in the bone marrow of resistant CML patients as compared to patients with an optimal response to TKIs treatment (CCyR).

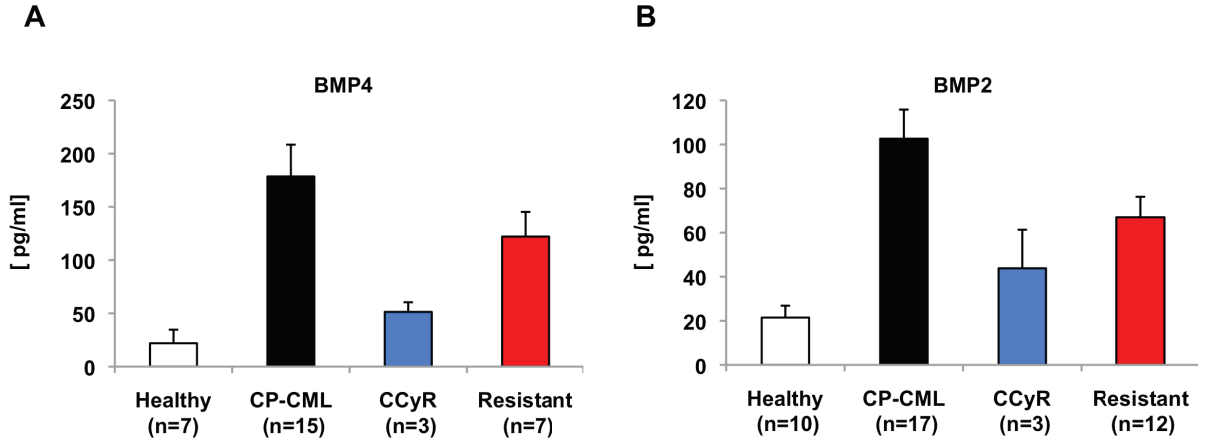

Figure 37 : Levels of BMP2 and BMP4 produced in the CML bone marrow microenvironment. (A) ELISA quantification of BMP4 and (B) BMP2 in BM plasma obtained from healthy donors, CP-CML patients at diagnosis, patients in complete cytogenetic remission (CCyR) or patients resistant to TKIs treatment. Results expressed in pg/mL represent the mean value ± SEM of the indicated number of analyzed samples.

2. Molecular alterations in BMP signaling elements in resistant CML cells

To evaluate whether the endogenous BMP pathway is specifically deregulated in CML patients resistant to TKI treatment, we compared the gene expression profiles of the main elements of the BMP pathway in CML cells at diagnosis prior to any treatment, using quantitative PCR (qPCR). After clinical follow-up of at least 2 years, the samples analysed at diagnosis were separated between sensitive and resistant to TKI treatment (Figure 38A). We

found that the expression of several components of the BMP pathway was deregulated in mature CD34$^-$ cells from resistant CML patients (Figure 38B). When compared to sensitive CD34$^-$ CML cells, *BMP2, BMPRIa, BMPRII, Bambi* and *Smad4* were significantly down-regulated while *Twist-1, BMP4* and *Foll* were significantly up-regulated. We then investigated if these changes could also affect immature CD34$^+$ CML cells. While *BMPRIa* and *Smad4* were significantly down-regulated, *Twist-1, BMP2, BMP4, FLRG, Smad1* and *Smad6* were up-regulated (Figure 38C). While this needs to be confirmed at protein level, the increase of both *BMP2* and *BMP4* expression in immature resistant CML cells could sign the activation of an autocrine loop for BMP production in these cells. These results indicate that the BMP pathway is altered in immature CD34$^+$ CML cells at all levels of the pathway.

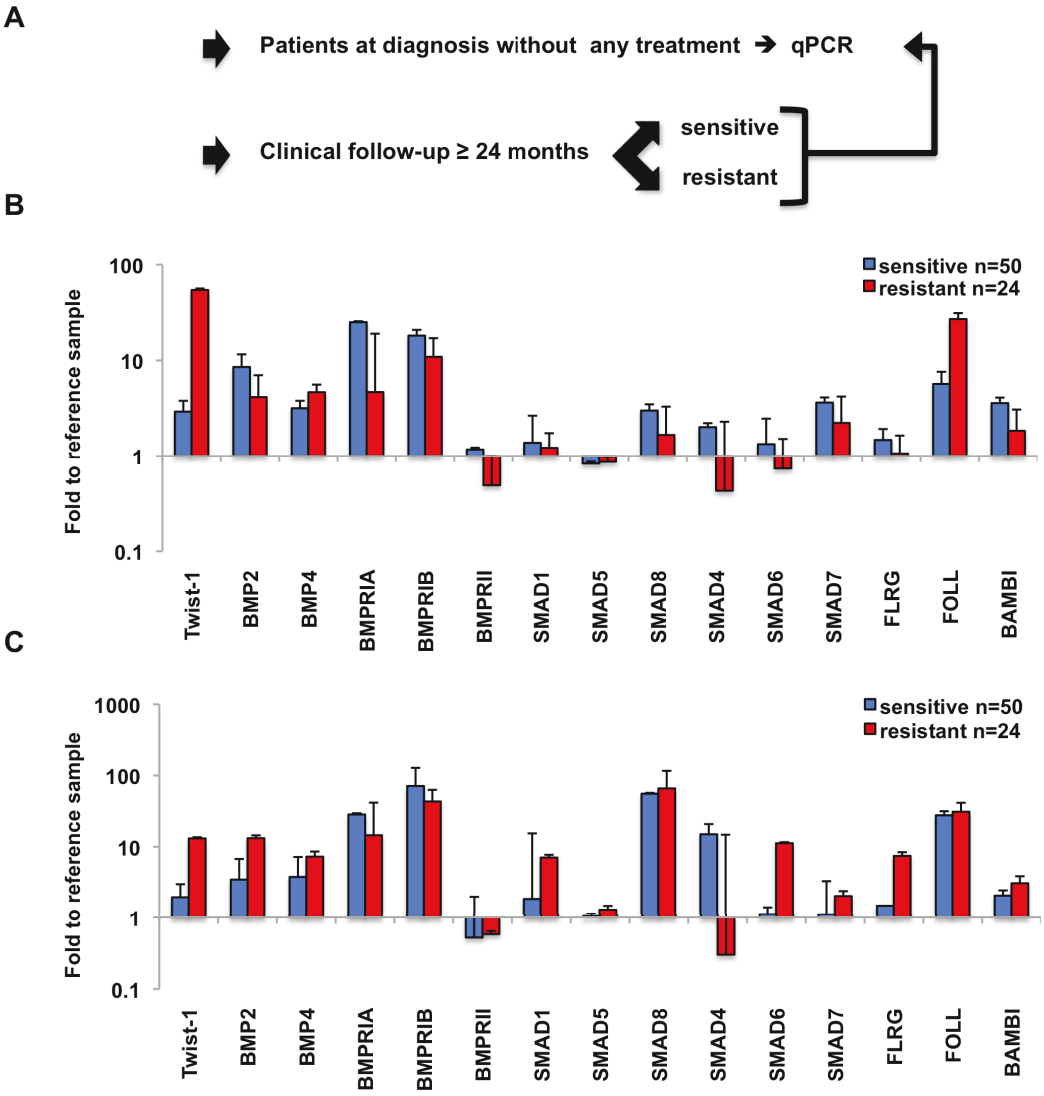

Figure 38 : Expression of BMP signaling elements in primary CML cells. (A) Gene expression (by qPCR) of *Twist-1* and BMP signaling elements in sensitive CML samples at diagnosis (blue bars, n=50) and resistant CML samples at diagnosis (red bars, n=24) for (B) CD34$^-$ cells or (C) CD34$^+$ immuno-selected hematopoietic cells. Results are expressed as fold change versus the reference value obtained for each gene using the same CD34$^-$ healthy donor sample.

Moreover, although the BMP signaling pathway seems to be profoundly altered with the resistance, in some cases these alterations are radically opposed between immature ($CD34^+$) and mature ($CD34^-$) compartments. For example, it is the case for *Smad6* gene, a specific inhibitor of the BMP signaling pathway.[491] Indeed, we observed a 10-fold increase of *Smad6* expression in $CD34^+$ cells from resistant patients compared to $CD34^+$ cells from sensitive patients (Figure 38), while almost a slight decrease was observed for *Smad6* expression in $CD34^-$ cells.

However, deregulation of *Smad6* in $CD34^+$ cells appeared more complex than we first realized. Indeed, when comparing its expression for each single patient, we observed an increased heterogeneity in *Smad6* gene expression levels in resistant patients (Figure 39A). We discriminated two groups of patients based on *Smad6* mean expression in $CD34^+$ cells isolated from healthy donors (Figure 39B). We then found that among 31 resistant patients samples, 7 over-express *Smad6* while 24 display a decrease of *Smad6* expression as compared to sensitive samples, suggesting the possibility of two different mechanisms involved in $CD34^+$ cells resistance to TKIs. Thus, a minority of resistant patients displayed a strong over-expression of *Smad6*, while most samples showed a significant decrease in expression of *Smad6* compared to the values observed in $CD34^+$ donors or patients sensitive to treatment (0.4 versus 1.6 and 1.3 respectively). Nevertheless, in all resistant patients, we observed an over-expression of *Twist1* in both mature and immature compartments, consistent with a previous report of our group. [445]

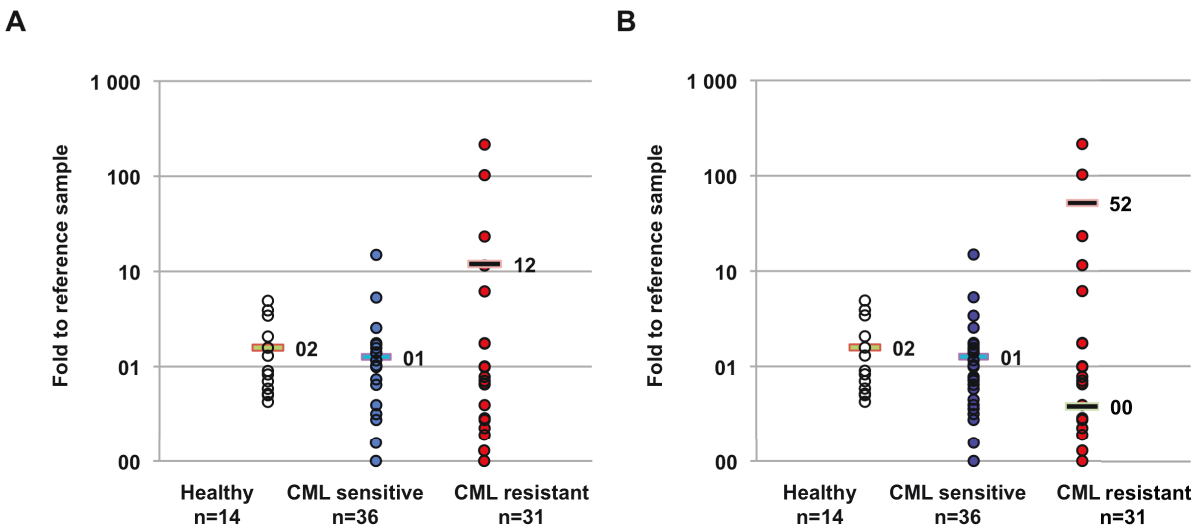

Figure 39 : Expression of *Smad6* in CD34$^+$ cells from healthy donors and CML patients. *Smad6* gene expression (by qPCR) in CD34$^+$ cells from healthy donors (white dots, n=14), sensitive CML samples at diagnosis (blue dots, n=36) and resistant CML samples at diagnosis (red dots, n=31). Each dot represents the value obtained for each individual sample. (A) The line and the associated value represent the mean of all the values obtained for a cell category. (B) For resistant CML patients at diagnosis only, the values were separated into two distinct sub-groups: a sub-group containing the samples with *Smad6* expression higher than the mean of sensitive CML samples, and a sub-group with the samples displaying Smad6 expression smaller than the mean of sensitive CML samples. The lines and the associated values represent the mean of all the values obtained for each sub-group. Results are expressed as fold change versus the reference value obtained for the same CD34$^-$ healthy donor sample.

3. Smad6 expression controls Imatinib sensitivity in KCL22 cells

We then evaluated the potential role of Smad6 expression on resistance of CML cells to Imatinib treatment. To this aim, we used the KCL22 CML cell line for which we dispose of sensitive (KCL22S) and resistant (KCL22R) clones to TKI treatment.[457] Each cell line was transduced with an empty vector (pCT) or a vector containing *Smad6* expressing sequence (pS6) and with a scramble vector (shCT) or a vector containing short hairpin RNA sequence directed against *Smad6* (shS6). After 2 days of culture, *Smad6* expression was controlled by qPCR and cells were incubated in the presence of different doses of Imatinib. After 3 days of culture, cell proliferation was determined by trypan blue staining. We then observed that Smad6 over-expression was associated with a decrease of cell proliferation in response to increasing doses of Imatinib in both sensitive and resistant cell lines (Figure 40A). Conversely, *Smad6* inhibition led to a decrease of KCL22 cells resistance to Imatinib treatment in sensitive and resistant clones (Figure 40B). Thus, the decrease of Smad6 expression observed in CD34$^+$ cells of most resistant patients compared to sensitive ones could be involved in their resistance to TKI treatment.

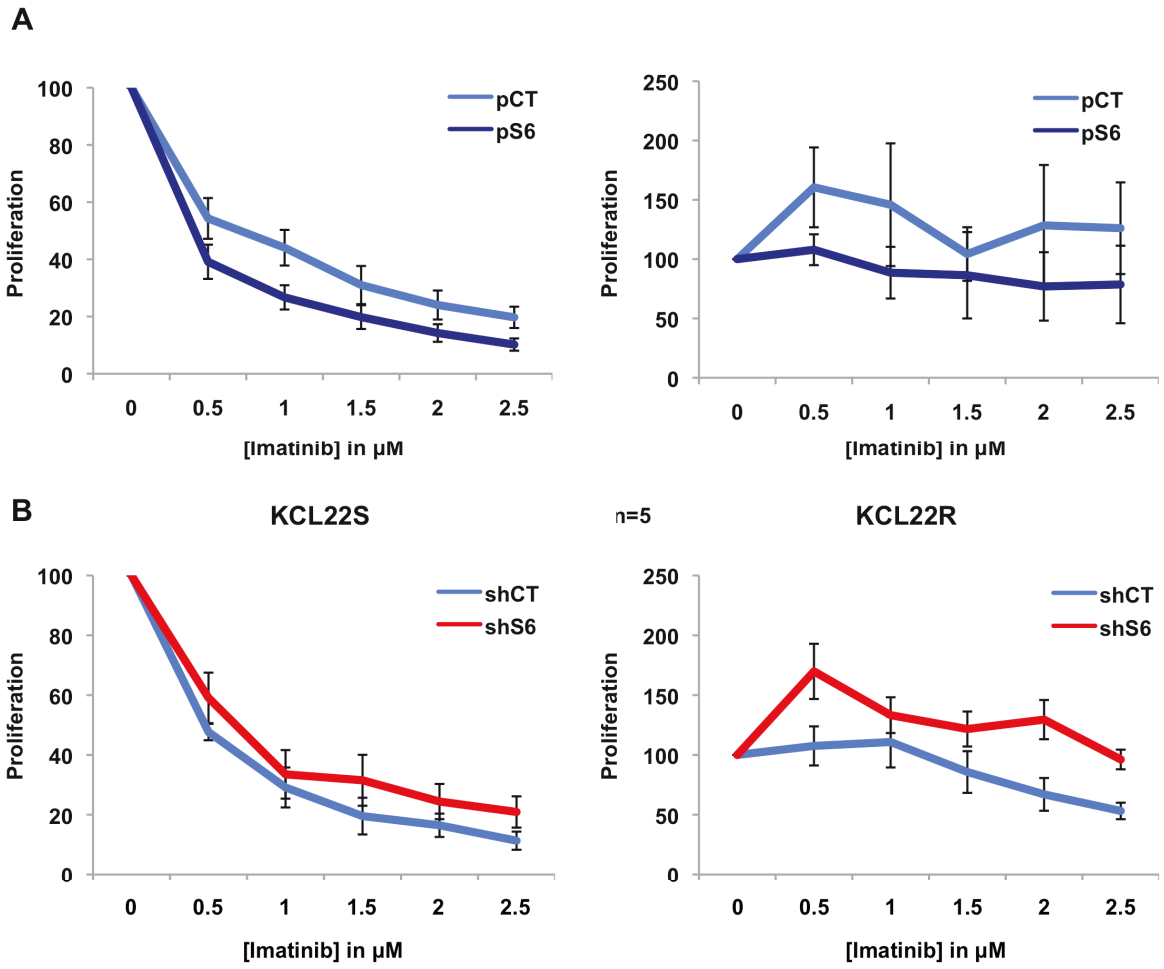

Figure 40 : Involvement of Smad6 in KCL22 CML cells response to TKIs. KCL22-sensitive (KCL22S) or KCL22-resistant (KCL22R) cells were transduced with (A) an empty vector (pCT) or a vector containing Smad6 expressing sequence (pS6) and with (B) a scramble vector (shCT) or a vector containing short hairpin RNA sequence directed against Smad6 (shS6). After 2 days of culture, cells were incubated in the presence of Imatinib at the indicated dose. After 3 days of culture, cell proliferation and viability were determined by trypan blue staining. The percentage of proliferation was determined by reference to the non-treated number of viable cells. Results represent the mean value ± SEM of n=5 experiments.

As we previously observed increased quantities of BMP2 and BMP4 in the BM of resistant CML patients, we then assessed the impact of BMP treatment on resistance of KCL22 cells to Imatinib treatment. After 3 days of culture in presence or not of BMP2 and BMP4, we observed a decrease in sensitive KCL22 cell proliferation in an Imatinib dose-dependent manner. However, no difference was observed between BMP-treated and untreated KCL22S cells (Figure 41A). Conversely, resistant KCL22 cells became even more resistant to increasing doses of Imatinib in presence of BMP2 and BMP4 (Figure 41B). Thus, these differences in the functional response to the same combination of soluble BMPs probably sign an altered molecular signaling between sensitive and resistant KCL22 cells.

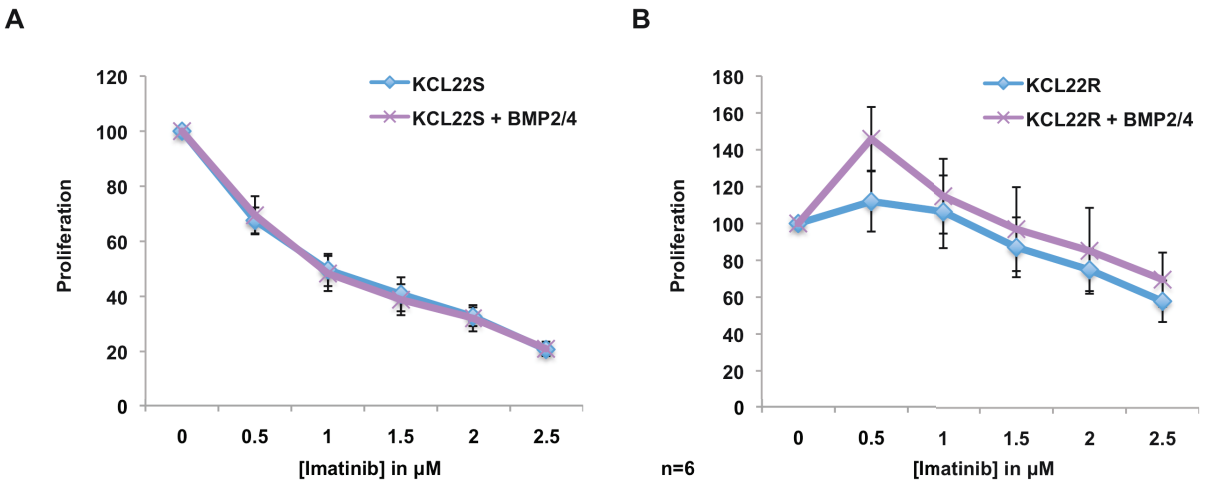

Figure 41 : Soluble BMP impact on KCL22 CML cells resistance to Imatinib. Sensitive (A) or resistant (B) KCL22 cells were incubated in the presence of BMP2 and BMP4 (50 ng/mL each). After 3 days of culture in presence of different concentrations of Imatinib, cell proliferation and viability were determined by trypan blue staining. The percentage of proliferation was determined by reference to the non-treated number of viable cells. Results represent the mean value ± SEM of n=6 experiments.

Our group previously identified *Twist-1* gene for his involvement in the resistance of immature cells CML.[445] Interestingly, depending on cell context, Twist1 is either considered as a BMP pathway target gene or as a regulator of this pathway.[446–449,490] To investigate whether the over-expression of *Twist-1* and deregulation of the BMP pathway elements are inter-dependent or independent of each other, we started to analyze the consequences of the expression of one of these elements on the expression of the other at the transcriptional level. We first modulated *Twist1* and *Smad6* genes expression and assessed their reciprocal impact in KCL22 cells by qPCR. After modulation of *Smad6* expression, we found that the expression of *Twist-1* was inversely correlated (Figure 42A). However, Twist-1 modulation had no impact on *Smad6* expression (Figure 42B). These results suggest that *Smad6* is able to control *Twist-1* expression in the CML context while the opposite is not true. Thus, the decrease of *Smad6* expression observed in most immature CML samples from resistant patients could be responsible of an increase in *Twist-1* expression also observed in resistant CML patients,[445] thus leading to an increased survival potential under TKI treatment. No significant effect was observed on *Bcr-Abl* expression after modulation of *Smad6* or *Twist1* expression (data not shown). However, these results need to be confirmed at the protein level and to be reproduced in more immature cell models.

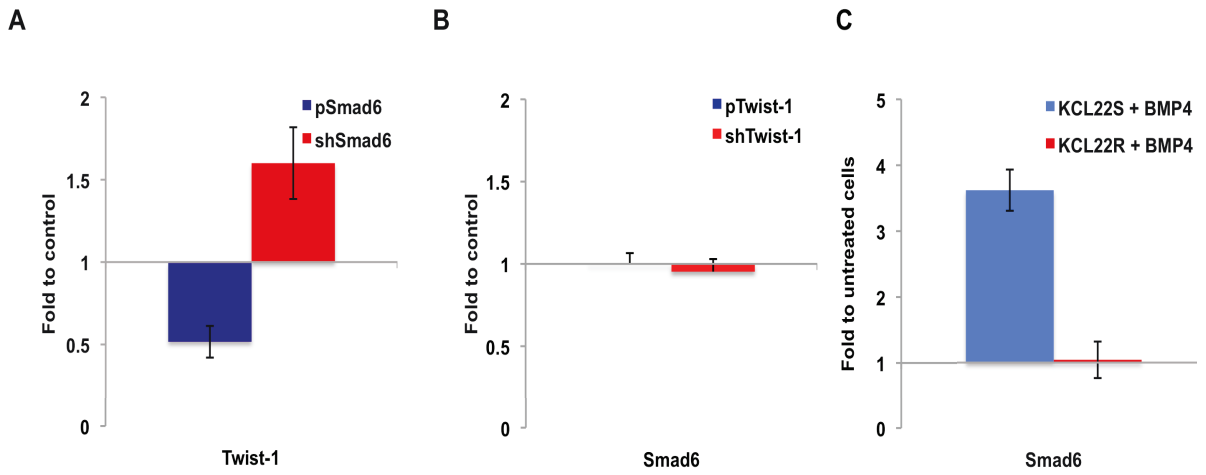

Figure 42 : Smad6 controls Twist-1 expression in KCL22 CML cells. (A) KCL22 cells were transduced with a vector containing *Smad6* expressing sequence (pSmad6) or with a vector containing short hairpin RNA sequence directed against *Smad6* (shSmad6) as well as with the corresponding control vectors. After 2 days of culture, *Twist-1* gene expression was assessed by qPCR. Results are expressed as fold change versus the control vector-transduced cells and represent the mean value ± SEM of n=3 experiments. (B) KCL22 cells were transduced with a vector containing *Twist-1* expressing sequence (pTwist-1) or with a vector containing short hairpin RNA sequence directed against Twist-1 (shTwist-1) as well as with the corresponding control vectors. After 2 days of culture, *Smad6* gene expression was assessed by qPCR. Results are expressed as fold change versus the control vector-transduced cells and represent the mean value ± SEM of n=4 experiments. (C) Sensitive (KCL22S) and resistant (KCL22R) KCL22 cells were cultured in presence or absence of 50 ng/mL BMP4 for 2 days. Smad6 gene expression was then assessed by qPCR. Results are expressed as fold change versus the untreated cells and represent the mean value ± SEM of n=4 experiments.

Furthermore, BMP signaling pathway regulation is a very complex process that includes negative feedback loops such as the increase of *Smad6* inhibitor expression in response to soluble BMPs signaling.[491] We then analyzed the status of this mechanism in sensitive and resistant KCL22 cell lines in response to BMP4 treatment. We observed an increase in the expression of *Smad6* inhibitor in response to a BMP4 treatment in sensitive KCL22 cells, while this is not the case for resistant KCL22 cells (Figure 42C). These preliminary results suggest the existence of a failure of the BMP pathway feedback mechanism to control *Smad6* expression in resistant KCL22 cells. Thus, *Smad6* inhibitor expression is not induced by BMP4 treatment, potentially leading to increased survival of resistant KCL22 cells to Imatinib treatment compared to sensitive KCL22 cells. However, these results have to be confirmed at the protein level, in primary samples from CML patients resistant to TKIs and will be completed by the analysis of the expression of *Smad6* in response to BMP2 treatment.

III. Discussion

Here, we report a significant increase in the availability of soluble BMP2 and BMP4 in the BM of resistant CML patients compared to CML patients in remission under TKI treatment. We also demonstrated that the BMP pathway is altered in cells from resistant CML patients compared to sensitive patients, and that some alterations seems to be specific of immature $CD34^+$ compartment. Indeed, we found that most of resistant CML patients display a decreased expression of *Smad6*, the expression of which seems to be involved in CML cells resistance to Imatinib treatment. Preliminary results suggest that loss of Smad6 expression could lead to the increased *Twist1* expression observed in immature cells from resistant CML patients,[445] thus leading to Imatinib resistance. Finally, we also demonstrated that resistant KCL22 cells were unable to increase their Smad6 expression level in response to soluble BMP4, like it is the case for sensitive KCL22 cells. This mechanism could potentially explain the differences observed in sensitive and resistant KCL22 cells when treated by soluble BMPs. Indeed, while BMP2 and BMP4 treatment had no effect on KCL22S cells resistance to Imatinib, they slightly increased KCL22R cells resistance upon Imatinib treatment. Our previous results demonstrated that alterations of intracellular BMP signaling pathway in CP-CML primary samples corrupt and amplify the response to exogenous high-levels of BMP2 and BMP4 thus participating to the amplification and maintenance of CML LSC in the chronic phase of the disease.[486] In line with this work, these preliminary results suggest that BMP pathway alterations could also be involved in the maintenance and resistance of CML LSC under TKI treatment.

In summary, we have demonstrated the existence of molecular alterations of the BMP pathway in immature resistant CML cells, as well as alterations in the quantities of soluble BMPs present in the tumoral niche of resistant patients. Finally, preliminary results suggest that loss of *Smad6* expression, observed in most resistant immature CML cells, combined with an increase of soluble BMPs in the BM niche could be involved in the resistance to Imatinib treatment. The analysis of the BMP pathway may therefore represent an interesting prognostic tool to assess the potential effectiveness of TKI treatment at diagnosis, allowing the design of drugs directly targeting the reservoir of LSC.

BCR-ABL increases cell stiffness in primitive CD34$^+$ CML cells

I. Introduction

Recent advances in micro- and nanotechnologies have enabled the capture of single cell mechanical properties and their modeling.[492–494] Atomic force microscopy (AFM) has proved to be a powerful, versatile tool to measure the mechanical properties of single living cells.[495,496] Cell mechanics are recognized to control critical cellular functions including migration and division,[496] and to be altered in some human diseases such as malaria,[497] diabetes[498] and cancer.[499] Indeed, several studies have suggested that tumor cells exhibit different mechanical properties compared to healthy cells.[286,287,289,290,294,500,501] As such, single cell mechanics may help to unravel the role of mechanical alterations in cell transformation processes and to quantify how malignant cells differ from healthy ones.[286] However, in most studies, experiments were carried out for different unrelated cell lines or few primary samples with often absence of normal counterpart.[499] Thus, the differences observed might only reflect a physiological heterogeneity in cell mechanical properties, as further confirmed by molecular analysis. Indeed, lack of a unique and reliable mechanical probing method is a severe hindrance to the comparison of these different studies. Chronic myelogenous leukemia (CML) arises from an hematopoietic stem cell transformation induced by a single reciprocal chromosomal translocation t(9;22) leading to the formation of the BCR-ABL oncogene.[487] CML represents a unique model to study Leukemic Stem Cell (LSCs) biology and to elucidate some of the mechanisms of cell transformation. As a myeloproliferative disorder, bone marrow cell density considerably increases during this disease,[502] and better knowledge of biophysical changes such as deformability, especially in LSCs, will improve our general understanding of this disease. However, unlike adherent cells, suspended hematopoietic cells pose a challenge for AFM because these cells tend to slip from under the cantilever tip when performing the nano-indentation.[298] In this study, we combined two different techniques to mechanically immobilize hematopoietic cells and to probe them. This also allowed us to study the effect of the microenvironment on cell stiffness. Finally, we performed a time-frequency analysis of force indentation curves to capture local variations of the cell stiffness between normal and CP-CML bone marrow purified CD34$^+$ cells.

II. Results

1. Atomic force microscopy and shear modulus extraction

Initially developed as a surface-imaging tool, AFM was further applied to stiffness measurements from nano- to micro-scales on adherent cells.[496,503] However, hematopoietic cells are usually suspended cells that tend to slip away from under the cantilever tip when performing the nano-indentation.[298] To avoid this phenomenon, we immobilized these cells using fibronectin-coated cover slips[504,505] or micro-fabricated wells[298]. Single living cells were then probed using an AFM cantilever controlled by a piezoelectric scanner and a laser tracking system (Methods). A sharp tip is moved back and forth towards the sample until a set point is reached and a force curve is generated (Figure 43A). The parameterization of force curves is most often performed assuming a linear elastic response of the sample as described by Hertz[506] or Sneddon[461] equations, depending on the shape of the cantilever tip (respectively spherical or pyramidal). Generalizing AFM force measurements to non-adherent cells was previously performed on hematopoietic cells, but the possible changes of the cells mechanical response during their deformation was not considered.[298] In this study, we revisited this assumption, considering that the elastic modulus was no longer a time-invariant quantity and that it may also include a viscous component. To capture changes in cell mechanical visco-elasticity during indentation, we developed an original analysis method inspired from previous studies in material sciences for pyramidal indentation of visco-elastic solids.[462] Given that the stress field is axially symmetric for a pyramidal indentation, the force-displacement curve is written as an integral equation that straightforwardly leads to the temporal shear modulus G(t) via a double derivation in time (Methods). The evolution of G, representative of cell stiffness, during cell indentation (I) was computed locally on the second derivative of the force using a wavelet-based analysis (Figure 43B).

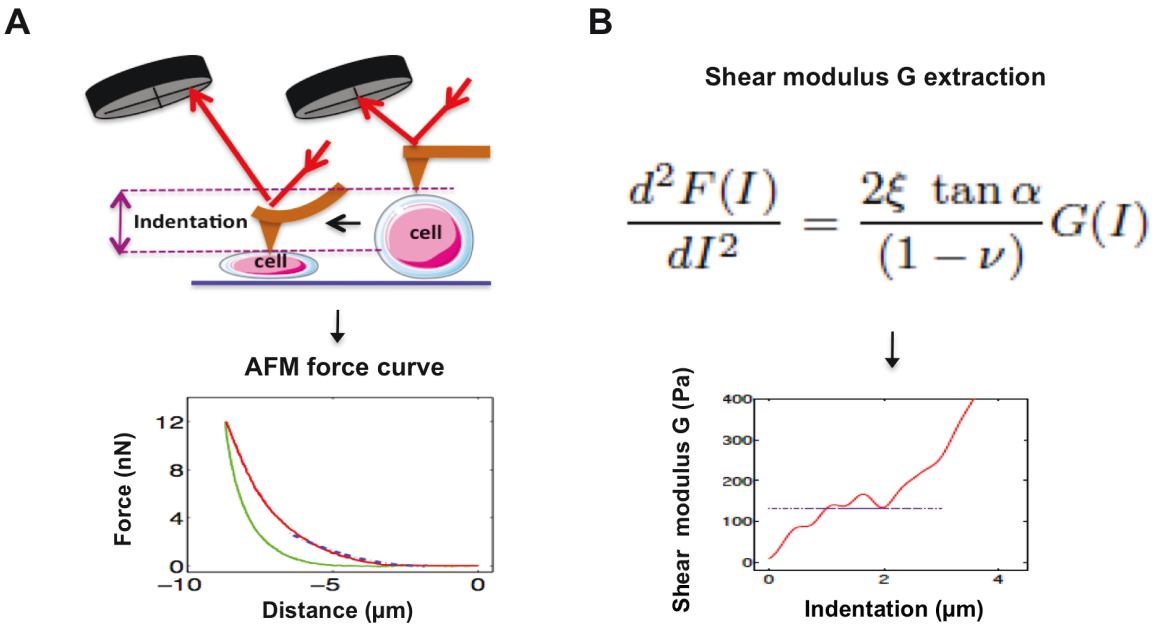

Figure 43 : Experimental setup and shear modulus extraction. (A) AFM force curves generation. The cells were allowed to settle into micro-fabricated wells, or to adhere on fibronectin-coated coverslips (Sigma) in culture treated plates (Fluorodish, Dutscher). Indentation was carried out at the center of each cell within 2 hours after removing cells from the incubator. (B) Shear modulus extraction from AFM force curves. The evolution of the shear modulus G during cell indentation is computed locally on the second derivative of the force F using a wavelet-based analysis. Each force curve was analyzed separately by a custom-written Matlab script.

We recorded 2 force curves from a suspended (Figure 44A) and an adherent TF1 cell (Figure 44B), displaying approach in red and retract in green. We used the Sneddon model to parameterize the approach force curves with a parabolic function with constant G (blue dashed line) within a 3 μm indentation range. We estimated the local curvature of the force curves using a second derivative of a Gaussian (width 400 nm) as analyzing wavelet. For the suspended TF1 cell, the transition to contact is smooth and the variation of G(I) is very progressive, reaching a plateau around 120 Pa that lasts about 2 μm, before an ultimate sharp increase (Figure 44A). For the adherent TF1 cell, the slope of the approach curve presents a discontinuity (from nearly zero to a finite value) when the cantilever tip comes in contact with the cell (Figure 44B). This discontinuity of the derivative of F appears as a hump in G of size given by the width of the analyzing wavelet. At larger indentation I, the G(I) curve reaches a plateau around 160 Pa that lasts over 2 μm before increasing again. The last part of the G(I) curves probably corresponds to the interaction of the cantilever tip with the nucleus, that appears to be stiffer than the surrounding cytoplasm. Even though the values of G(I) are not very different on the plateaus before the nucleus deformation, the way the

suspended and adherent cells respond to the mechanical stress is very different. The former responds as a volume body with a visco-elasticity that increases progressively with the deformation while the later responds as a stiffer cortex impeding the penetration of the cantilever tip. These robust observations of change in local shear modulus put into light the need of revisiting the analysis of immature hematopoietic cell mechanical response.

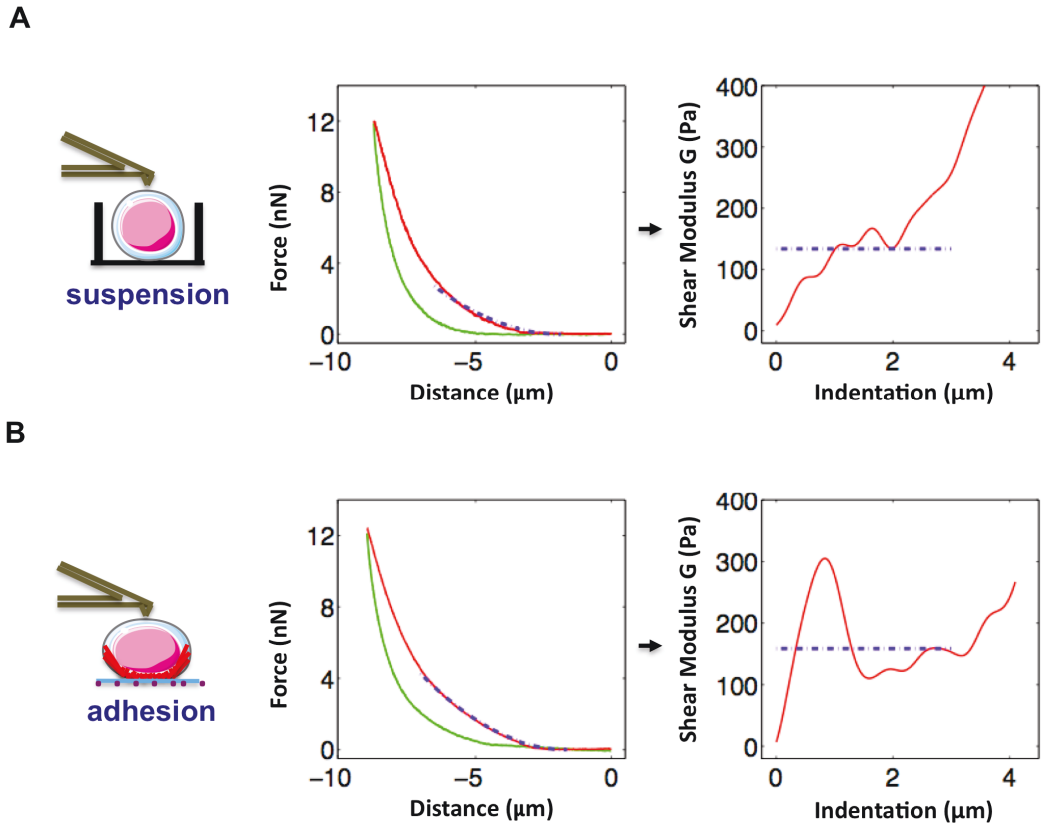

Figure 44 : Shear modulus extraction from AFM force curves on immature hematopoietic TF1 cells. (A) Approach (red) and retract (green) curves on a suspended TF1 cell. The blue dashed line corresponds to a parameterization of F(Z) with a parabolic function. G(I) curve was computed from the second derivative of the force curve, obtained with a fixed size (width 400 nm) second-order analyzing wavelet. (B) The same curves were obtained for a TF1 cell in adhesion to fibronectin.

2. Statistical analysis of the dynamical response of hematopoietic cells

To perform a statistical analysis of the dynamical response of soft hematopoietic cells under mechanical stress, we considered both the shear modulus G and the indentation I. Indeed, if we take the mean of G computed with a parabolic parameterization of the force curve, we may over- or under-estimate drastically G depending on the interval we use for this parameterization. Thanks to our wavelet-based analysis of G evolution during cell indentation, we computed histograms of G values on different intervals of I for a set of 30 force curves recorded on an adherent TF1 cell (Figure 45A). The wide distribution of the

histogram displaying all the G values did not allow to obtain a single G value that could be characteristic of cell stiffness. We then divided I range into 4 intervals to obtain 4 different histograms (Figure 45A). The histograms obtained for intervals II [1 µm, 2 µm] and III [2 µm, 3 µm] display a marked peak, meaning that within these ranges of indentation, the G curves cross a plateau, where the mechanical properties of the cell are invariant. Conversely, the histograms obtained for intervals I [0 µm, 1 µm] and IV [3 µm, 4 µm] display a wide range of G values. The differences observed at specific intervals, but not for all of them, support the idea to consider not only a mean value or a specific indentation, but the shear modulus evolution for all indentations inside the cell. As heterogeneity in cell size distribution could bias cell stiffness comparisons, we normalized I, dividing it by the diameter (D) of each individual cell. Finally, cell mechanical profiles were obtained by constructing histograms with two entries, respectively the shear modulus G, the indentation to cell diameter (I/D) ratio and the frequency (F) of shear modulus signal for each considered I/D ratio (Figure 45B). The histograms were normalized by the total number of points in the G(I) curves. We observed an increase of G during AFM indentation.

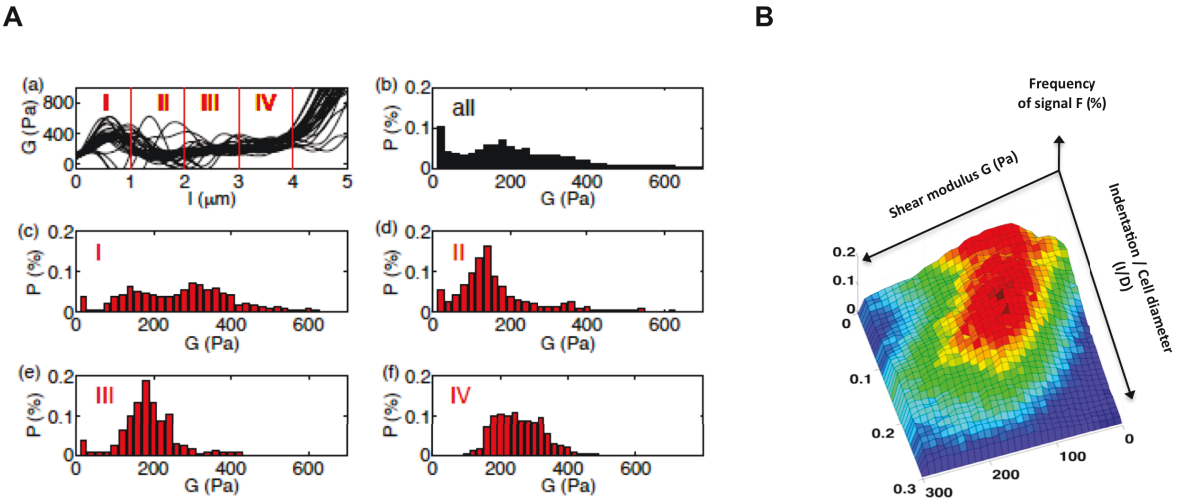

Figure 45 : Cell mechanical profiles construction. (A) Histogram reconstruction from 30 G(I) curves reconstructed from AFM forces curves recorded on an adherent TF1 cell. G(I) curves are displayed with the four intervals used for the histogram computation. Histogram of G values were computed over different indentation intervals: all [0µm, 5µm], I [0 µm, 1 µm], II [1 µm, 2 µm], III [2 µm, 3 µm] and IV [3 µm, 4 µm]. (B) Cell mechanical profiles were obtained by constructing normalized histograms with three entries, respectively the shear modulus G, the indentation to cell diameter (I/D) ratio and the frequency (F) of shear modulus signal for each considered I/D ratio.

3. BCR-ABL expression leads to an increased cell stiffness in primitive CD34$^+$ CP-CML cells

Using this strategy, we compared the mechanical properties of bone marrow CD34$^+$ cells immunoselected from 4 allogeneic donor samples and 4 chronic phase (CP) CML samples of patients at diagnosis and prior to any treatment (Figure 46A). While the mechanical profiles of the 4 allogeneic donor or the 4 CP-CML samples are heterogeneous, the differences between healthy and CP-CML CD34$^+$ cells are striking. Indeed, G values appear to be more dispersed and generally more elevated for CP-CML samples than for healthy counterparts (Figures 46B and C).

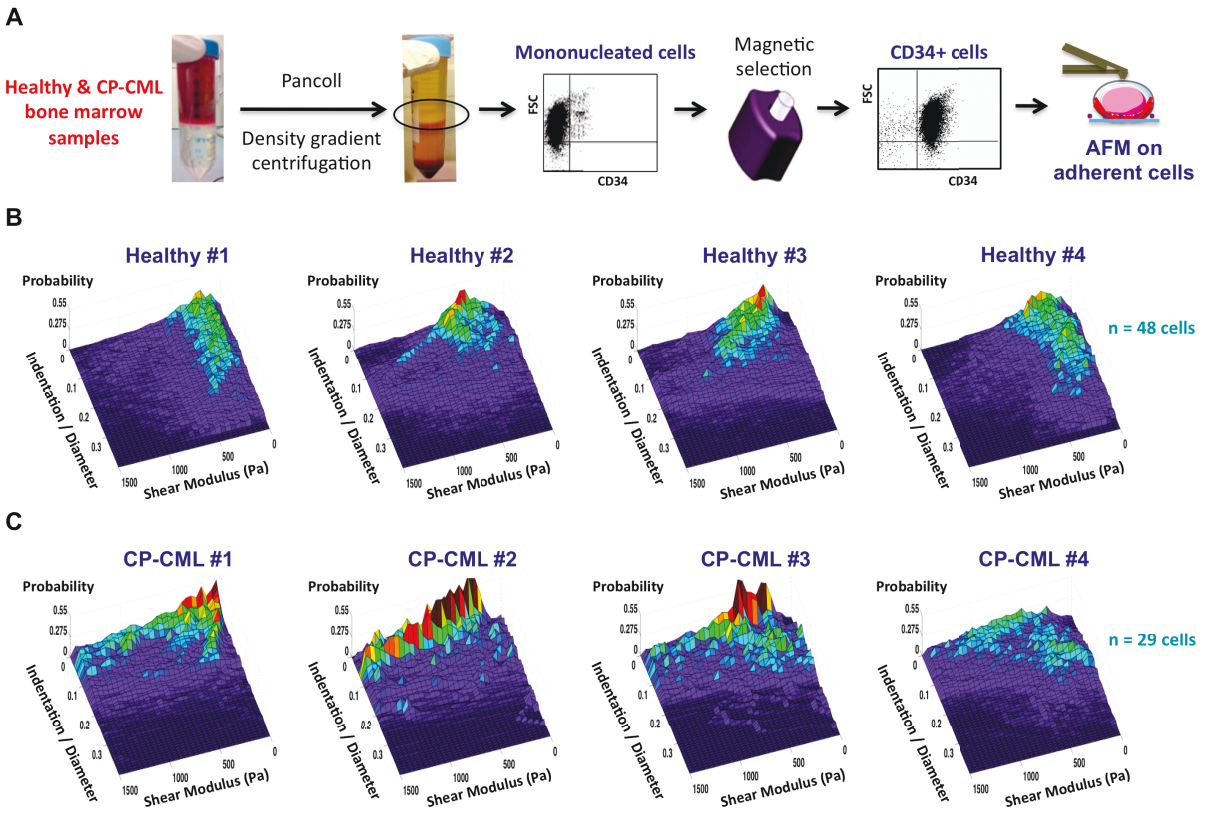

Figure 46 : Comparative analysis of cell stiffness. (A) Bone marrow samples were obtained from CML patients at diagnosis and from healthy allogeneic donors. Mononuclear cells were separated using a Pancoll gradient and were then subjected to CD34 immunomagnetic separation. CD34$^+$ cells were then allowed to adhere on fibronectin-coated coverslips before being probed by AFM. Cell mechanical profiles were computed for (B) healthy (n=48 cells from 4 different healthy samples) and (C) CP-CML (n=29 cells from 4 different CP-CML samples) bone marrow CD34+ cells.

To visualize these differences, we computed a single mechanical profile by pooling together the 4 different samples for each condition, and observed an alteration of the mechanical properties of CD34$^+$ bone marrow CP-CML cells as compared to normal counterparts, visualized in Figure E by the shift of the peak toward the left (Figure 47).

Indeed, while G reaches a plateau around 500 Pa for indentations up to 10% in normal $CD34^+$ cells, G values increase up to 1500 Pa in CP-CML $CD34^+$ cells for the same indentations. This reflects a higher resistance of leukemic cells in response to cantilever applied force at a similar depth.

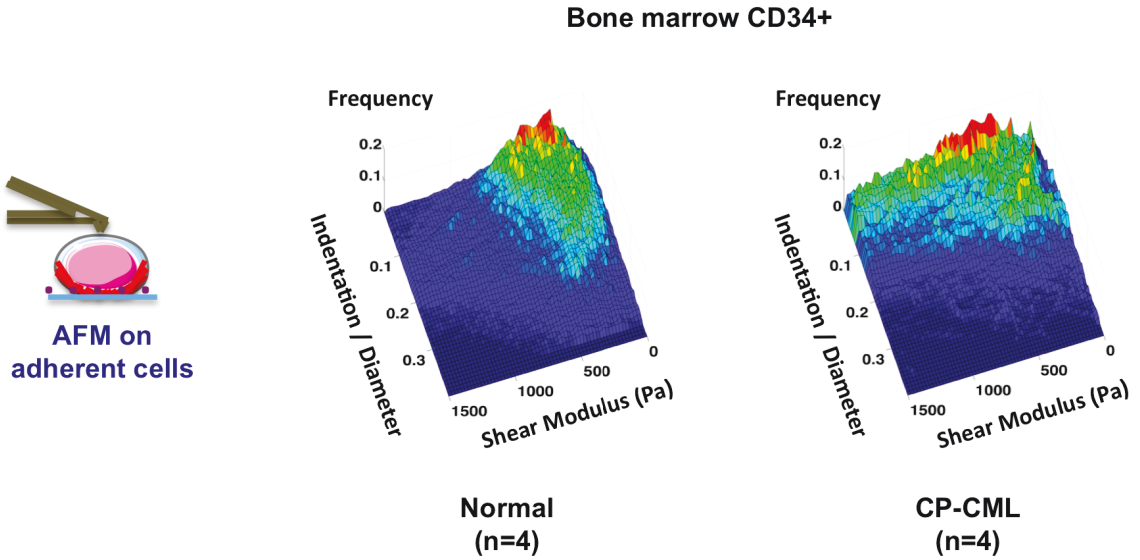

Figure 47 : Cell stiffness is increased in primary CP-CML cells. Cell mechanical profiles were computed for healthy (n=48 cells from 4 different healthy samples) and CP-CML (n=29 cells from 4 different CP-CML samples) bone marrow $CD34^+$ cells.

To evaluate if these differences are related to cell transformation, we used a relevant CML model reproducing early steps of leukemic transformation, based on the retroviral transduction of BCR-ABL in the human $CD34^+$ TF1 cell line.[486] Using this model, we assessed the impact of BCR-ABL expression on TF1 cell stiffness by performing AFM on cells immobilized in micro-fabricated wells (Figure 48A) or by adhesion to fibronectin (Figure 48B). While no differences were found between the mechanical profiles of wild type (TF1wt) and GFP-transduced (TF1-GFP) TF1 cells, BCR-ABL-transduced (TF1-BCR-ABL) TF1 cells became stiffer compared to TF1wt or TF1-GFP cells in both suspended or adherent conditions. Indeed, the shear modulus observed for suspended TF1-BCR-ABL cells spreads to larger values with the emergence of a lateral shift around 300 Pa for indentation comprised between 10% and 20%. The original frequency peak of these distributions around 100 Pa is not shifted but its amplitude is decreased by 70% (Figure 48A). However, the differences observed in adherent conditions are even more striking. These results, visualized by the shift of the frequency toward the left from 150 Pa up to 200 Pa for indentations comprised between 10% and 20%, indicates that BCR-ABL expression alone is sufficient to increase immature TF1 cells stiffness, particularly when these cells are cultured in adhesion

to fibronectin (Figure 48B). We also observed that the G values of TF1-BCR-ABL cells are more broadly distributed than for TF1-GFP cells in suspended and adherent conditions, reflecting a higher heterogeneity in the transformed cells' mechanical properties.

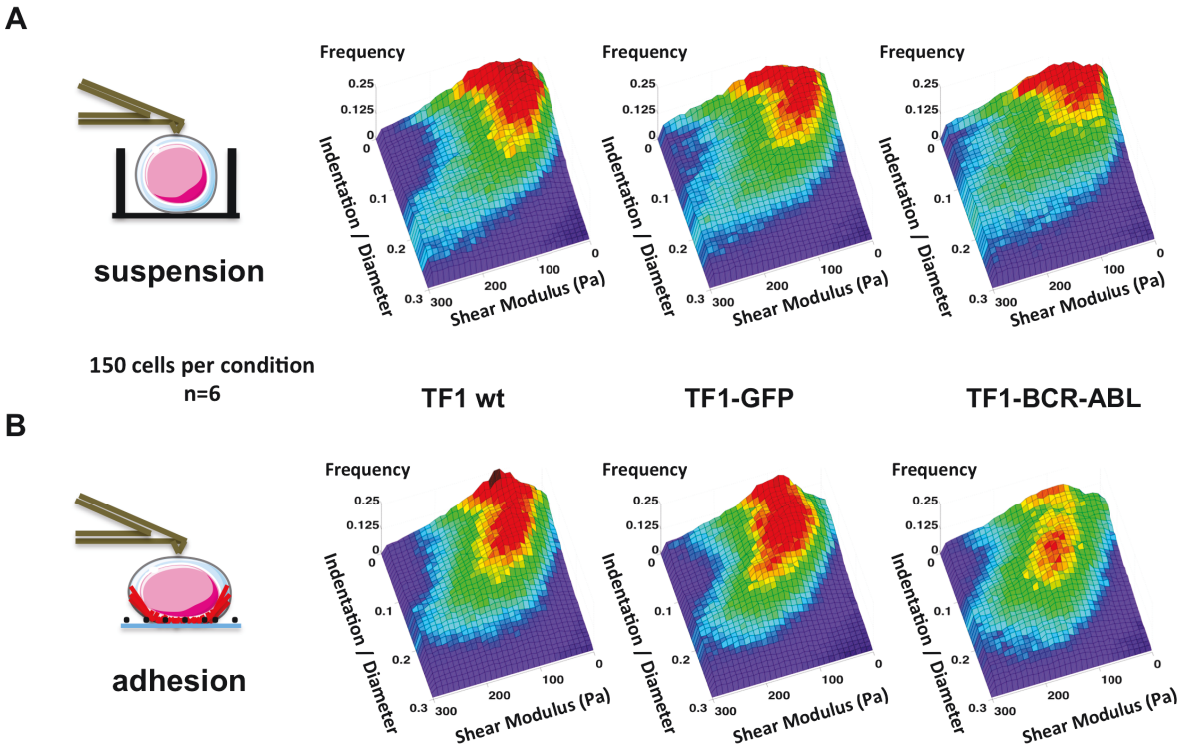

Figure 48 : BCR-ABL expression increases TF1 cell stiffness in adhesion to fibronectin. Cell mechanical profiles were computed for wild type, GFP-transduced and BCR-ABL-transduced TF1 cells (A) immobilized in micro-fabricated wells and (B) in adhesion to fibronectin (n=150 cells each from 6 independent experiments).

4. The microenvironment controls cell stiffness

The differences observed after BCR-ABL transduction in TF1 cells, particularly in adhesion to fibronectin, support a crucial role for cell microenvironment in the control of their mechanical properties. Interestingly, we also observed an increase of cell stiffness in adhesion to fibronectin for TF1wt and TF1-GFP cells, but not as important as for TF1-BCR-ABL cells (Figure 48A and B). Whereas G reaches a plateau around 100 Pa for indentations up to 20% in suspended TF1 cells, in adherent TF1 cells G do not saturate but rather keep increasing, as illustrated by the bend toward the left observed at larger indentations. This shows that the adherent cells are stiffer on average than suspended cells and that this stiffness increases with the indentation (Figures 48A and B). Thus, cell mechanical properties are probably also dependent on their interactions within a defined microenvironment and on their geographical location. In CML particularly, immature $CD34^+$ cells are able to detach from their bone marrow microenvironment to circulate through the

blood vessels,[507] another environment with different interactions and no adhesion. This suggests that the mechanical properties of these cells extracted from different locations could be different. To test this hypothesis, we compared the mechanical properties of $CD34^+$ cells extracted either from the peripheral blood or from the bone marrow of a single CP-CML patient. We then observed an increased stiffness in $CD34^+$ cells isolated from bone marrow as compared to those located in peripheral blood of the same CP-CML patient (Figure 49). While G do not exceed 700 Pa for indentations up to 10% in peripheral blood $CD34^+$ cells, its value increase up to 1500 Pa for bone marrow $CD34^+$ cells at similar indentations. These results demonstrate that both intrinsic (BCR-ABL expression) and extrinsic (adhesion to fibronectin) parameters are involved in cell stiffness control.

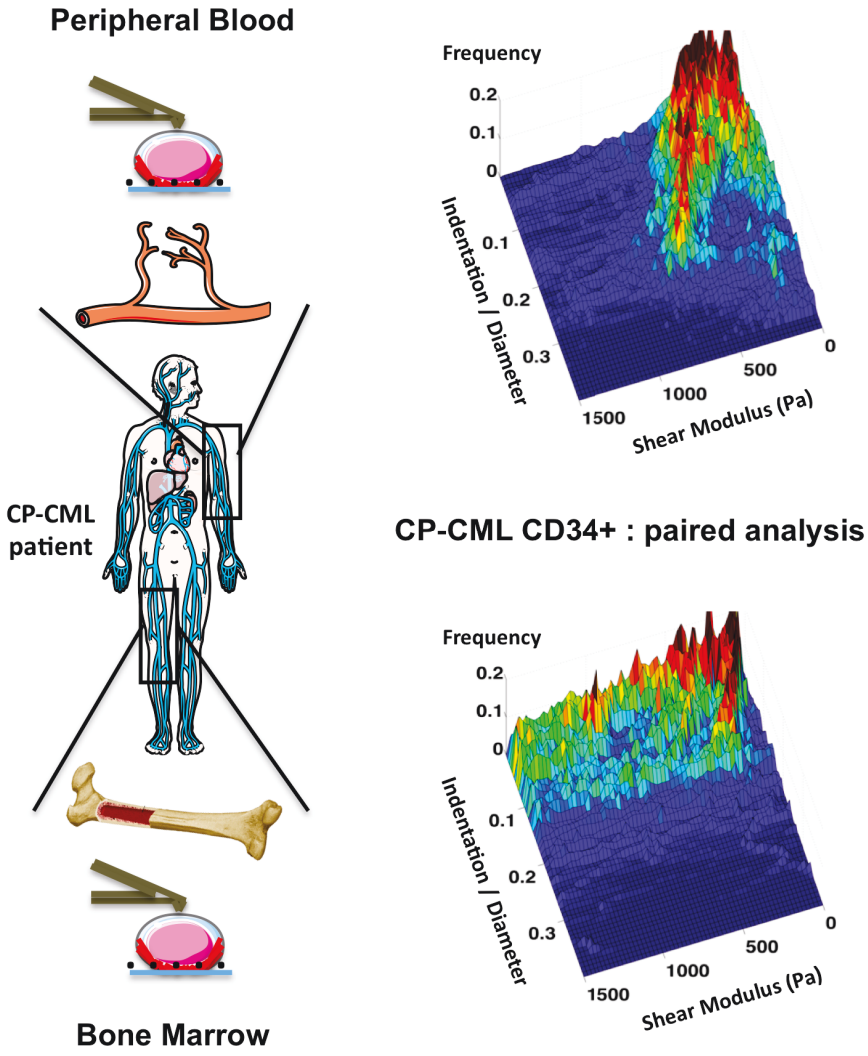

Figure 49 : Cell stiffness is controlled by their environment. Cell mechanical profiles were computed for $CD34^+$ cells extracted from the peripheral blood (n=15 cells) or the bone marrow (n=12 cells) of a unique CP-CML patient.

5. Alterations of cell mechanical properties are correlated with modifications of F-actin cytoskeleton organisation

Interestingly, in transformed cells, BCR-ABL has been demonstrated to bind actin filaments (F-actin),[508] one of the major determinants of cell mechanical behavior,[509] and to induce its redistribution into punctate, juxtanuclear aggregates.[108] In addition, the binding to F-actin seems to be involved in the transforming ability of BCR-ABL.[108] To investigate the impact of BCR-ABL transduction on actin cytoskeletal network of TF1 cells, the localization of GFP-BCR-ABL or control GFP proteins was examined by immunofluorescence after phalloidin-rhodamine treatment to detect F-actin in suspended and adherent cells. In suspended parental or TF1-GFP cells, F-actin was localized in the cortical cytoskeleton. However, in TF1-BCR-ABL cells, juxtanuclear F-actin aggregates were found in almost 30% of the cells in addition to the cortical F-actin staining (Figure 50). These structures were induced by BCR-ABL as they were rarely observed in the parental or TF1-GFP cell lines. No co-localization was observed between cortical F-actin and GFP or GFP-BCR-ABL proteins, mainly detected in the cell nucleus with a diffuse cytoplasm staining.

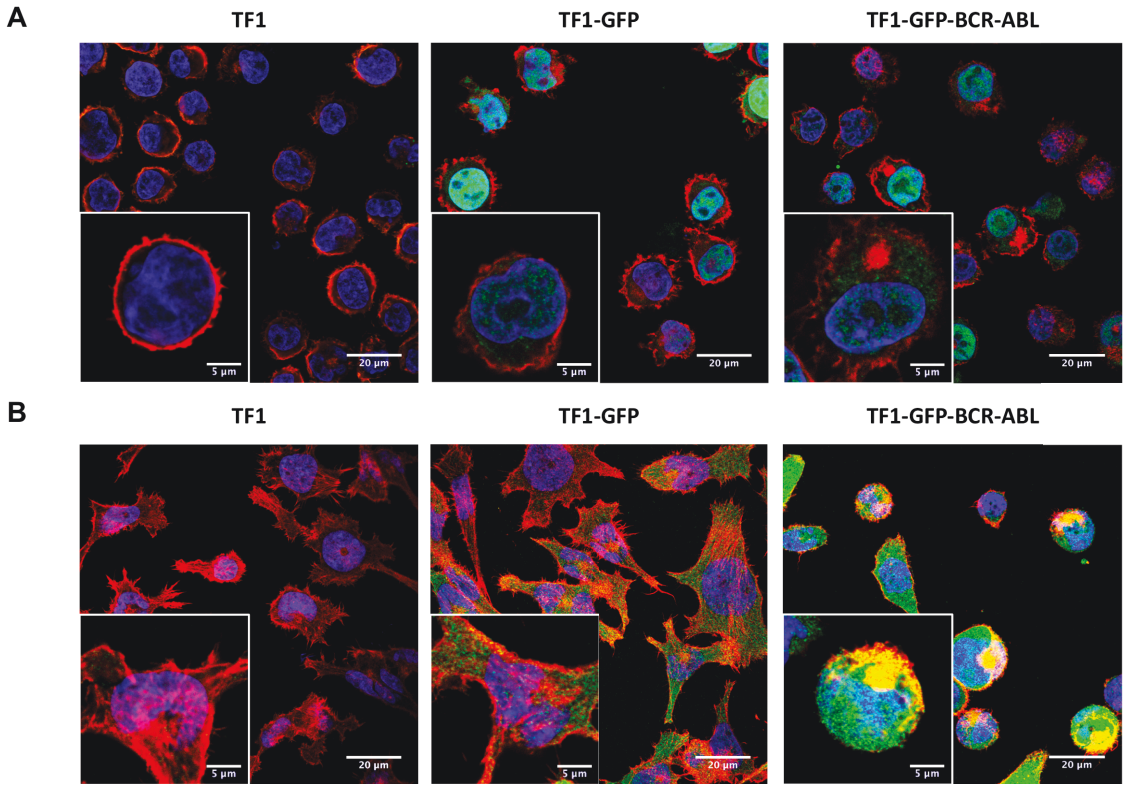

Figure 50 : BCR-ABL alters F-actin distribution in TF1 cells. Filamentous actin (F-actin) was labeled with phalloidin-rhodamin (red), GFP signal was amplified using a rabbit polyclonal anti-GFP antibody-Alexa Fluor 488 conjugate (green) and the nuclei were labeled with DAPI (blue). Immunofluorescence images representative of 6 different experiments were taken using a confocal microscope on TF1wt, TF1-GFP and TF1-GFP-BCR-ABL cells either in a suspended state (A) or in adhesion to fibronectin (B). Scale bar 20 µm. Magnification examples are presented in lower left panels for each condition. Scale bar 5 µm.

When cells were allowed to adhere to fibronectin, the formation of actin stress fibers was observed in their cytoplasm (Figure 50B). This indicates that actin polymerization is correlated with increased cell stiffness in adherent cells when compared to non-adherent cells. While all cell types displayed rounded shapes in suspension, adherent cells appeared more spread and flattened, except for TF1-BCR-ABL cells that predominantly kept a rounded morphology even in adhesion (Figures 50A and B). Consistent with those data, TF1-BCR-ABL cells contained fewer actin stress fibers compared to parental or TF1-GFP cells in adhesion. As expected, GFP proteins did not co-localize with F-actin in TF1 cells. They exhibited a diffuse localization in both the nucleus and the cytoplasm. In contrast, GFP-BCR-ABL proteins were localized in both the nucleus and the cytoplasm and a small fraction of the total protein co-localized with actin stress fibers as well as juxtanuclear F-actin aggregates, still present in adherent conditions. Altogether, these results demonstrate that BCR-ABL oncoprotein leads to the alteration of actin cytoskeletal network and consequently of the mechanical properties of TF1 cells.

The adhesion was also accompanied by a flattening of the cells. We use a quantitative phase microscopy (QPM) device recently developed in our laboratory[472] to compare the shape and index of these cells in suspended and adhesive conditions. We observed TF1 cells with a very regular rounded shape with small protrusions on their surfaces (Figure 51A). QPM provides a quantitative and non-intrusive estimation of the cell flattening during their adhesion. The cross-sections show that this flattening is limited to about half the original cell diameter, probably because these cells have a nucleus that occupies a large part of the cell volume that limits their deformation. This is consistent with the observation on the force curves at large indentation of a steep increase of the shear modulus when the tip of the cantilever comes in interaction with the nucleus. In the TF1 cell line, BCR-ABL transduction leads to a profound modification of the cell internal organization since TF1-BCR-ABL cell sizes can reach twice the size of TF1wt or TF1-GFP cells (Figure 51B). This is illustrated in the QPM images of TF1-BCR-ABL cells as compared to TF1-GFP cells. Despite the drastic increase of the diameter (from 14.6 µm ± 1.5 to 19 µm ± 3, computed from a sample of 25 QPM images) of these cells, their optical path length (OPL) does not change much (from 0.52 µm ± 0.05 to 0.53 µm ± 0.1), meaning that the volumic ratio nucleus/cell is likely decreasing, leaving more space for the cytoplasm. However, no differences in cell sizes were observed between primary $CD34^+$ cells obtained from healthy donors and CP-CML patients.

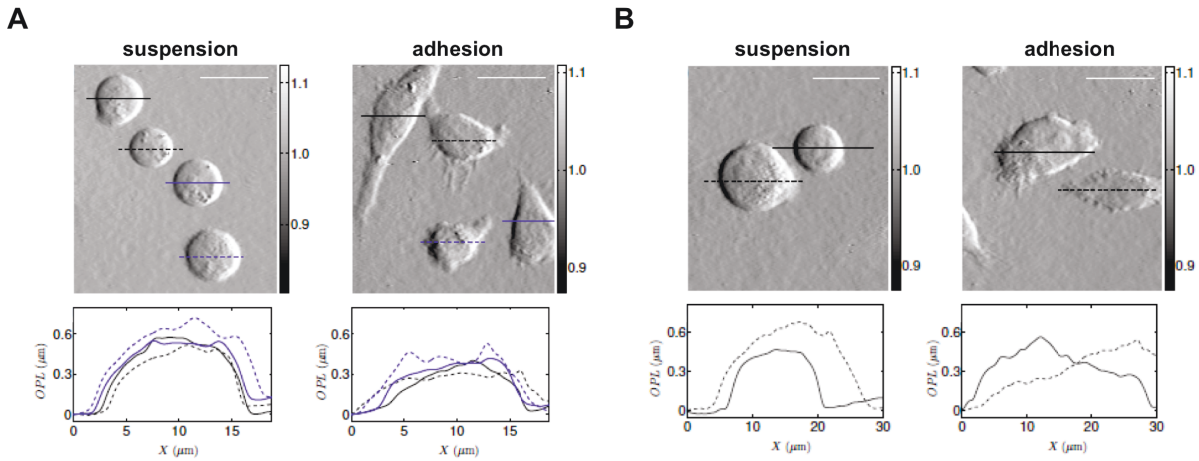

Figure 51 : Quantitative phase microscopy (QPM) characterization of TF1 cells. (A) Image of the phase derivative ∂φ/∂x of suspended and adherent TF1-GFP cells. The optical path length (OPL) was computed from the phase derivative on the sections marked by the black and blue lines. (B) Image of the optical phase derivative ∂φ/∂x of suspended and adherent TF1-BCR-ABL cells. The optical path length (OPL) was computed from the phase derivative on the sections marked by the black and blue lines. Scale bar 20 µm.

III. Discussion

Hematopoietic cells have been identified as the softest cells of the human body.[278] We noticed a great variety of mechanical responses of TF1 cells. This variability led us to repeat the force curves on a large set of cells for each cell type. When comparing the different cell types mechanical properties, we also observed that primary $CD34^+$ cells are much more stiffer as compared to $CD34^+$ TF1 cells.

Culturing immature hematopoietic cells on fibronectin-coated surface may also preferentially select cells with higher adhesion capabilities. In CML particularly, BCR-ABL is known for its ability to decrease progenitor cell adhesion to the BM stroma.[510] By performing adhesion assays, we demonstrate that BCR-ABL transduction has no effect on the percentage of adhesion of TF1 cells on fibronectin-coated surfaces (Figure 52A). However, combining immunofluorescence and AFM, we show that adhesion changes qualitatively since TF1-BCR-ABL cells loose their ability to mature adhesion by the formation of actin stress fibers, in link with the presence of F-actin aggregates in 30% of the cells (Figure 52B).

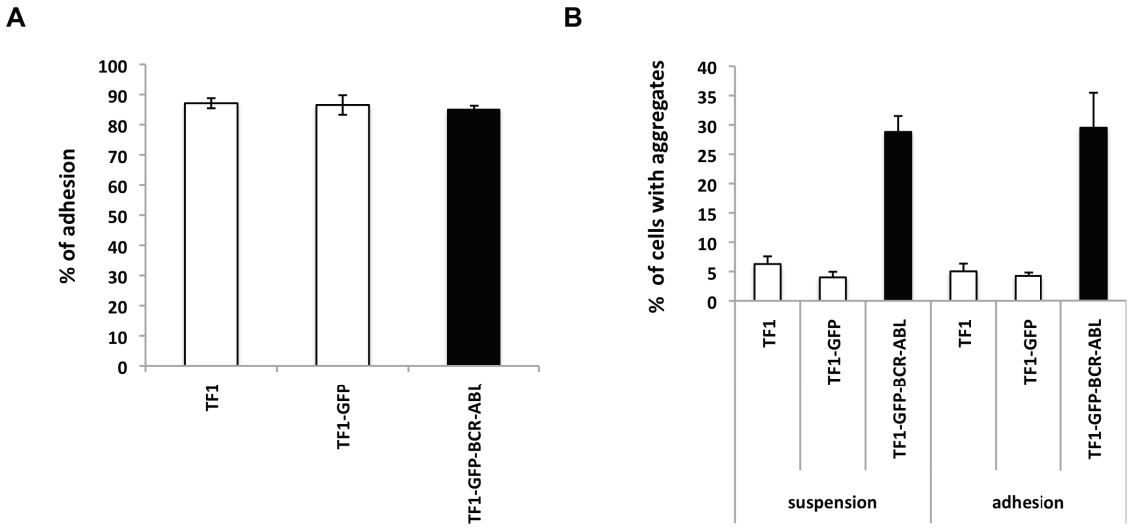

Figure 52 : Effects of BCR-ABL transduction on adhesion and F-actin aggregates formation in TF1 cells. (A) 5.10^4 cells/well of each type TF1wt, TF1-GFP and TF1-GFP-BCR-ABL labeled with 5 µM calcein-AM were allowed to adhere for 1 hour to fibronectin-coated 96-well plates. Fluorescence was quantified before and after the removal of non-adherent cells. The mean percentages of adhesion: %adhesion = 100.(Fluorescence of adherent fraction/fluorescence of whole cells) and their standard errors (SEM) are computed from a set of n = 8 experiments. (B) Suspended TF1wt, TF1-GFP and TF1-BCR-ABL cells were either cytospined onto untreated glass slides or allowed to adhere on glass cover slips coated with fibronectin for one hour before being labeled for F-actin with phalloidin-rhodamin. F-actin aggregates were then scored using a confocal microscope on suspended and adherent cells. The mean percentage of cells containing aggregates and their standard errors ± SEM are computed for a set of n=6 experiments.

By analyzing more than 27000 AFM force curves out of 900 cells, we reveal for the first time the alteration of the mechanical properties of immature CML cells upon BCR-ABL oncogene expression. The wavelet-based extraction method of the shear modulus introduced here encompasses other parametrization methods, allowing a direct scale matching of the analyzing wavelet with the local force-indentation response curvature. We demonstrate that cancer cells not only become stiffer but that their mechanical response to stress changes, reinforcing their cortical tension and decreasing their ability to form actin stress fibers. We further reveal an increase of the stiffness of suspended hematopoietic progenitors when adhered to fibronectin. These results are consistent with the appearance of actin stress fibers, confirming the central role of F-actin in cell mechanical properties. As immature CML cells resistant to TKIs are likely to reside in bone marrow, our data open new perspectives in the understanding of how leukemic cells can withstand constraints exerted by their microenvironment to promote their survival and drug escape. Such a mechanical approach could be used to develop new diagnosis or prognosis tools for the early detection of cancer.

Cell confinement modulates the growth of BCR-ABL$^+$ CD34$^+$ cells through up-regulation of Twist-1 expression

I. Introduction

Over the past few years, there has been increasing interest in understanding SC biology,[489] particularly as it relates to tumor initiation and progression.[84,511] Microenvironmental signals, including biochemical and biomechanical factors, are known to control SC fate.[512] Even though most studies in the literature place an emphasis on growth factors and cytokines, it has been evidenced that mechanical signals also significantly influence cell fate.[513] Recent advances in micro- and nano-technologies have opened a new field of research devoted to cell mechano-biology.[514,515] It has now been established that cells can sense their physical surroundings through mechano-transduction,[516] by translating mechanical forces and deformations into biochemical signals such as changes in genes expression or by activating diverse signaling pathways.[517] In turn, these signals can adjust cellular and extracellular structure.[518] This mechano-sensitive feedback modulates many critical cellular functions, ranging from differentiation,[519] proliferation,[281] quiescence,[280] self-renewal[277] or apoptosis, and is crucial for organ development and homeostasis.[279,520] However, cell mechanics can also be altered in some human diseases such as cancer.[499] Consequently, defects in cellular mechano-transduction processes could contribute to the tumor phenotype. Indeed, recent studies have demonstrated that mechanical stress can cooperate with genetic lesions to promote cancer progression.[315] This could be particularly relevant in the bone marrow (BM), a viscous tissue located in the bones that contains hematopoietic stem cells (HSCs). Due to its confinement by bones, the BM has a unique mechanical environment that can be affected by external factors[452], such as blood flow, physiological activity, aging or disease. Thus, changes in the bone marrow mechanical environment may also affect the fate of resident HSCs. This could prove especially important in the tumor context, where uncontrolled proliferation of cancer cells entails their compaction within their microenvironment,[322] resulting in constant mechanical stress. However, little is known about how this mechanical stress influences tumor cell behavior. Chronic myelogenous leukemia (CML) arises from a HSC transformation following the formation of the BCR-ABL oncogene by a reciprocal chromosomal translocation (t9;22).[487] Therefore, CML represents a unique model to study leukemic stem cells biology and to elucidate some of the mechanisms of cell transformation. We demonstrated that immature CML cells possess a stiffer cortex as compared to healthy cells. Thus, the alteration of the cells intrinsic mechanical properties could lead to the alteration of their functional response to external

mechanical stress. Recently, investigators have attempted to elucidate these mechano-transduction pathways through controlled biomechanical experiments.[459,460] Different studies have demonstrated that cells submitted to mechanical stress will express specific « mechano-sensitives » genes, like *Twist-1*[274,315,521] or BMP pathway elements.[243,366–368,376] Numerous systems have been designed by physicists to dynamically measure and control forces on single cells, such as atomic force microscopes,[503] micro-plates[522] or micromanipulators.[523] While the aforementioned studies have provided great insight into cell biomechanical properties, functional and molecular biology protocols allowing advanced analysis of confined cells are challenging at single cell level. Here, we use a method based on the gentle application of a confining slide on the cultured cells using a modified multi-well plate (in collaboration with M. Piel, Institut Curie Paris).[273,458–460] Because the confinement is applied on a population of cells, statistical data are easy to obtain, and because confinement can be released, the cell material is available for further biological analysis. In this study, we subjected normal and leukemic immature cells to defined compression using this unique cell-confining device.

II. Results

1. Hematopoietic cells confinement

To apply a controlled force on a population of hematopoietic cells, we adapted a system recently developed for adherent cells confinement.[273] Briefly, the cells were allowed to adhere onto fibronectin-coated wells before being compressed by a gentle application of a confinement glass slide (Figure 53A). The diameter of the confinement slides was chosen to fit inside 24-wells plates, allowing the testing of different conditions simultaneously (Figure 53B). The distance between the slide and the culture substrate is controlled by polydimethylsiloxane (PDMS) (relatively stiff biocompatible silicone rubber) microspacers molded under the confinement slide. Thus, the cells' height is precisely imposed by defining the height of the microspacers, ensuring a geometric confinement (Figure 53C).[458] The rigid glass slide provides homogeneous confinement with no long-range deformation and the soft material (PDMS) ensures the robustness of the confinement by absorbing the local substrate inhomogeneities. To evaluate the impact of BCR-ABL expression on cell response to an external mechanical stress, we confined GFP-transduced and BCR-ABL-transduced human $CD34^+$ TF1 cells (Figure 53D). As previously described, this model reproduces the main features observed in primary immature chronic phase (CP) CML cells.[486] Microspacers high enough to avoid cell confinement (30 µm) were used as control. Un-confined suspended and

fibronectin-adherent cells were also tested. The cells were confined for 3 days before being collected for further analysis. We first evaluated their viability and proliferation using trypan blue staining and cell counting. We observed a slight decrease of cell viability (5%) in presence of confining slides, consistent with the surface occupied by PDMS microspacers, suggesting that 5% of the cells were crushed under microspacers. While cell confinement had no impact on TF1-GFP cell viability, 10 µm confinement slightly increased TF1-BCR-ABL cell viability, that was then decreased upon increased confinement (Figure 53E).

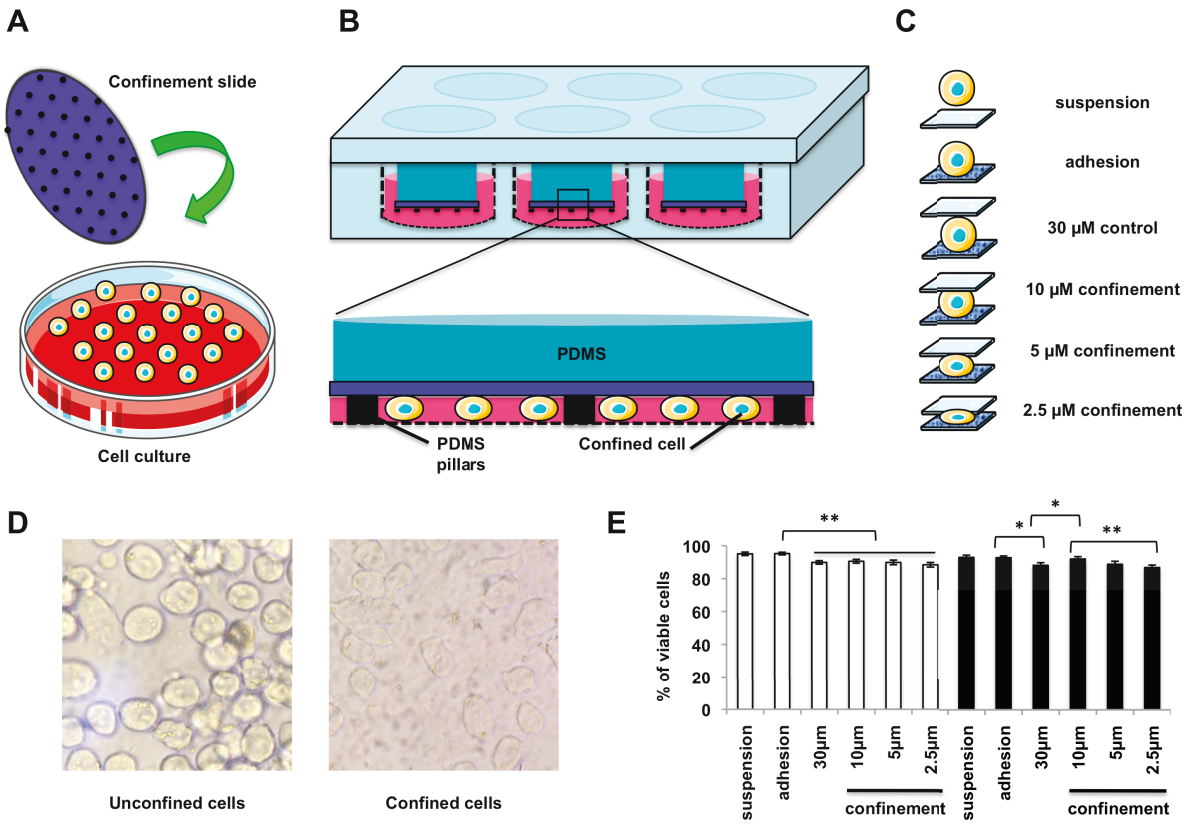

Figure 53 : Experimental setup for hematopoietic cells confinement. (A) Confinement slides are glass cover-slips covered by polydimethylsiloxane (PDMS) microspacers. They can be applied reversibly on the cell culture to provide a homogeneous confinement. (B) The lid of a multiwell plate is modified by introducing large PDMS pillars to hold the confinement slides. These pillars have the height of the plate, which can be slightly increased with a thin PDMS layer. When the multiwell plate containing adherent cells and culture medium is closed with the modified lid, the confinement slides are applied on the cells adhering to the bottom of the wells. Large pillars that are slightly higher than the well depth deform and apply pressure on the confinement slides. (C) To prevent hematopoietic cells to slip away when applying the confinement slides, we immobilized them using fibronectin-coated wells. By defining the height of the microspacers, respectively 10µm, 5µm and 2.5µm, we ensure a precise geometric confinement of hematopoietic cells. Microspacers high enough to avoid cell confinement (30µm) are used as control. (D) Images of control and confined TF1 cells. (E) Cell viability was evaluated in TF1-GFP (open bars) and TF1-BCR-ABL (closed bars) confined cells using trypan blue staining and cell counting. Results are expressed as the % of viable cells. Results are presented as the mean ± SEM of n=10 experiments. *p<0.05; **p<0.005 indicate differences between two conditions.

2. BCR-ABL expression modulates TF1 cells proliferation in response to mechanical signals

Strikingly, these differences of cell viability seem to reflect differences of cell proliferation. Indeed, while cell confinement had no impact on TF1-GFP cell proliferation, 10 µm confinement led to an increase of TF1-BCR-ABL cell proliferation that was then lost upon increased mechanical confinement (Figure 54A). These results were confirmed using KCL22 CML cell line (data not shown) and were also correlated with the expression of Ki67 proliferation marker determined by flow cytometry (Figure 54B). We then performed cell-cycle analysis to assess the potential impact of mechanical stress on cell division. Although cell confinement had no effect on TF1-GFP cells repartition between G_0/G_1, S and G_2/M phases (Figure 54C), it significantly impacted TF1-BCR-ABL cells. Indeed, TF1-BCR-ABL cells displayed a shift from G_0/G_1 phase to S and G2/M phases upon 10 µm confinement. Conversely, stronger confinements reversed this effect, as illustrated by an increase of the percentage of cells in G_0/G_1 phase at the expense of S and G_2/M phases at 2.5 µm confinement (Figure 54D). Together, these results demonstrate that BCR-ABL expression increased the sensitivity of TF1 cells to mechanical compression by affecting cell-cycle distribution thus leading to a force-dependent altered proliferation.

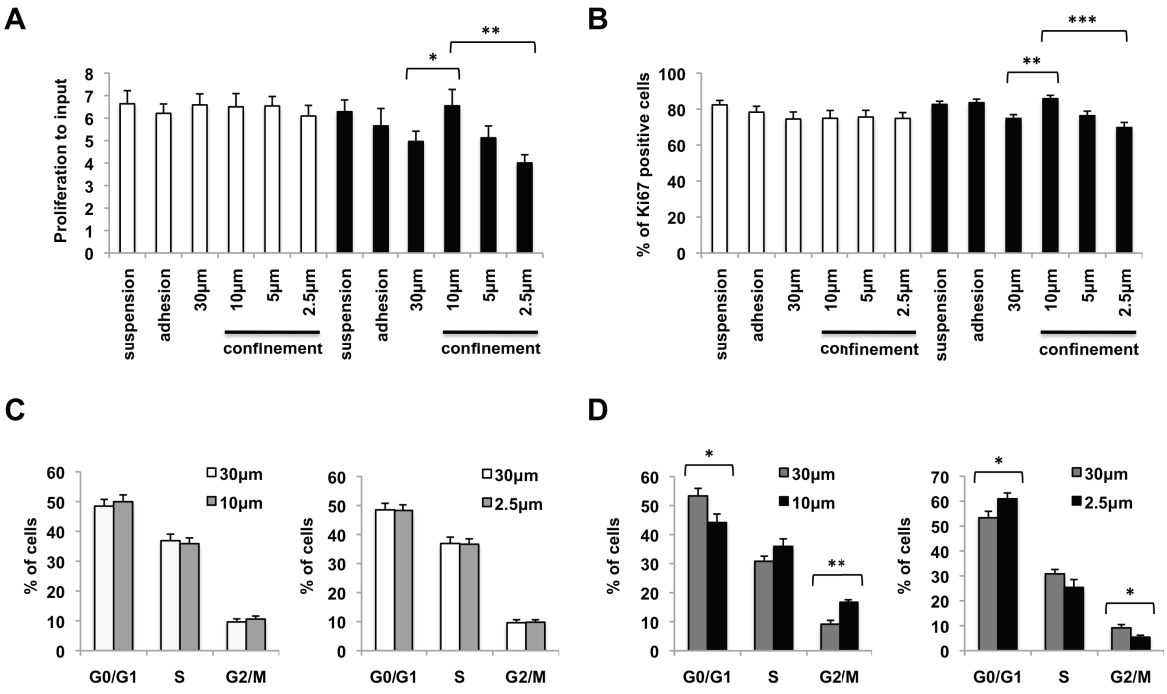

Figure 54 : Effect of mechanical stress on cell proliferation. (A) Cell proliferation was evaluated in wild-type (open bars) and BCR-ABL-transduced (closed bars) confined TF1 cells using trypan blue staining and cell counting. Results are expressed as the proliferation ratio that is the ratio of cell counts to the number of input cells. (B) Phenotypic analysis of the proliferation marker Ki67 was performed using flow cytometry on a Facscalibur® cell analyzer (Becton Dickinson) and gated for viable cells. Cell-cycle profiles of (C) TF1-GFP

and (D) TF1-BCR-ABL confined cells were determined using the Watson model on Flowjo after propidium iodide staining. Cell repartition between G_0/G_1, S and G_2/M cell-cycle phases was determined for 10 µm and 2.5 µm confinement compared to 30 µm control. Results are presented as the mean ± SEM of n=10 experiments. *p<0.05; **p<0.005; ***p<0.0001 indicate differences between two conditions.

We then assessed the impact of cell confinement on bone marrow stromal cells using HS27A cell line. To do so, we used the same conditions as for TF1 cells confinement, except that we did not need to perform fibronectin coating to induce stromal cells adhesion. After 3 days of confinement, cells were collected to evaluate their viability and proliferation using trypan blue staining and cell counting. While cell confinement had no impact on cell viability (Figure 55A), it led to a strong and force-dependent reduction in cell proliferation (from 50% at 5 µm confinement to 30% at 2.5 µm confinement) (Figure 55B). The same results were also observed using HS5 stromal and BMEC-1 micro-vascular cell lines (data not shown). Thus, these results demonstrate that stromal cells are much more sensitive to mechanical signals as compared to hematopoietic cells.

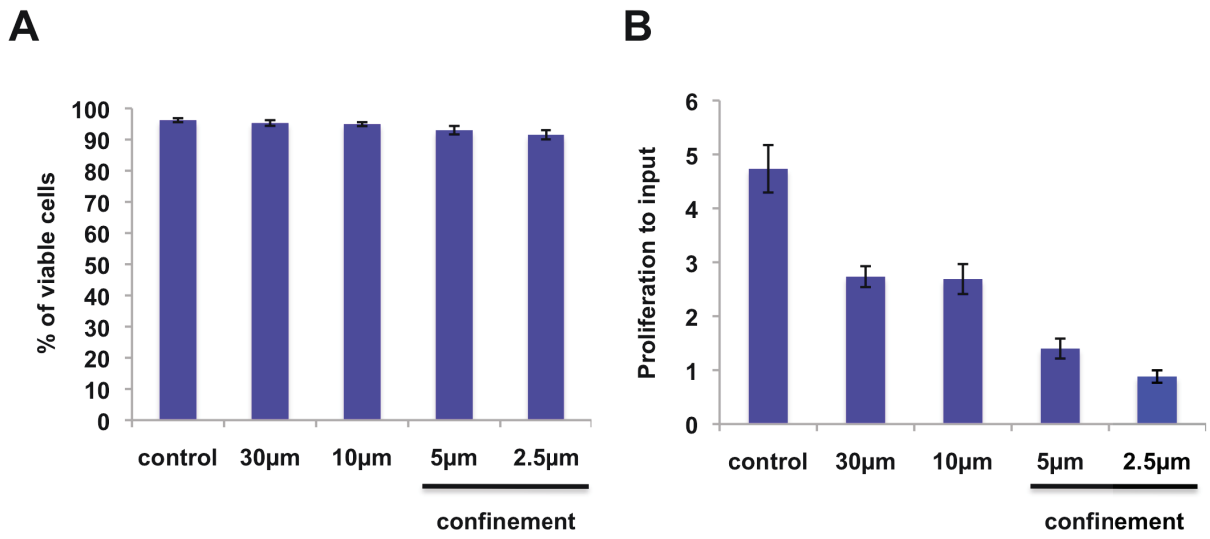

Figure 55 : Effect of mechanical stress on stromal cell proliferation. Cell viability (A) and proliferation (B) were evaluated in confined HS27A cells using trypan blue staining and cell counting. Results are expressed as the % of viable cells or as the proliferation ratio that is the ratio of cell counts to the number of input cells. Results are presented as the mean ± SEM of n=6 experiments.

We next evaluated the impact of cell confinement on immature properties of TF1 cells. We first demonstrated that cell confinement had no impact on immature CD34 marker expression at the membrane of both TF1-GFP and TF1-BCR-ABL cells using flow cytometry (Figure 56A). We further used the CFC assay to determine the impact of cell confinement on progenitor cell content. While BCR-ABL transduction led to a significant increase in colony numbers, no significant differences were observed after cell confinement (Figure 56B). We then scored the colonies as erythroid or myeloid (Figure 56C) and late or early (Figure 56D),

and observed an increase in the number of late myeloid colonies after BCR-ABL-transduction, but no impact of cell confinement.

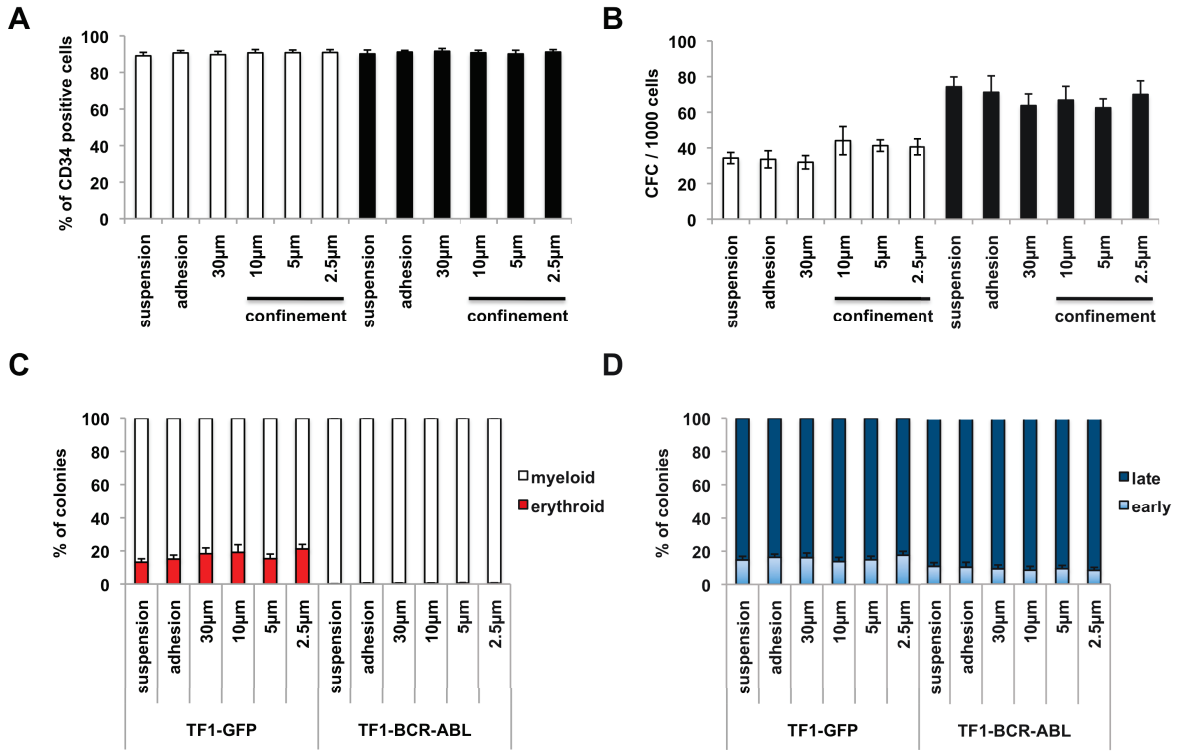

Figure 56 : Effect of mechanical stress on progenitor content. TF1-GFP (open bars) and TF1-BCR-ABL (closed bars) cells were confined for 3 days. (A) Phenotypic analysis of the immature hematopoietic cell marker CD34 was then performed using flow cytometry on a Facscalibur® cell analyzer (Becton Dickinson) and gated for viable cells. (B) The progenitor content of confined cells was analyzed by the clonogenic CFC assay. Results are expressed as the total CFC colonies per $1x10^3$ seeded cells and represent the mean ± SEM of n=10 experiments. The content of treated cells in distinct hematopoietic progenitor categories was determined by scoring the colonies as (C) erythroid or myeloid, and (D) late (one to two clusters for erythroid colonies, below $1x10^3$ cells/colony for myeloid colonies) or early. Values of colony subtypes are expressed as the percentage of the total number of colonies.

3. Cell confinement induces Twist-1 expression in TF1-BCR-ABL cells

We further analyzed by qPCR the expression of a set of genes (BMP pathway and *Twist-1* gene) involved in the transduction of mechanical signals in different cell types, and previously identified for their crucial role in immature CML cells functions. While cell confinement had no impact on *BMP2*, *BMP4*, BMP receptors and *Smad6* expression (data not shown), we observed a differential expression of *Twist-1*, a bHLH embryonic transcription factor also described as an oncogene,[524] under cell confinement. Interestingly, *Twist-1* expression in $CD34^+$ cells has been described as a new predictive factor of the response of CML patients to TKI treatment.[445] Here, we found that *Bcr-Abl* expression alone was able to induce a 12-fold increase of *Twist-1* expression in TF1 cells (Figure 57A). Whereas cell

confinement had no effect on *Twist-1* expression in TF1-GFP cells, 10 µm confinement led to a additional 2-fold increase of *Twist-1* expression in TF1-BCR-ABL cells compared to non confined cells. However, only a small and non-significant increase of *Twist-1* expression was observed for higher confinements (5 µm and 2.5 µm confinement). As BCR-ABL transduction was directly able to induce *Twist-1* expression in TF1 cells, we then measured the levels of BCR-ABL expression after cell confinement, and demonstrated that mechanical stress had no impact on BCR-ABL expression in TF1 cells (Figure 57B). In addition, Twist-1 modulation under cell confinement was confirmed at the protein level by western-blot analysis (Figure 57C).

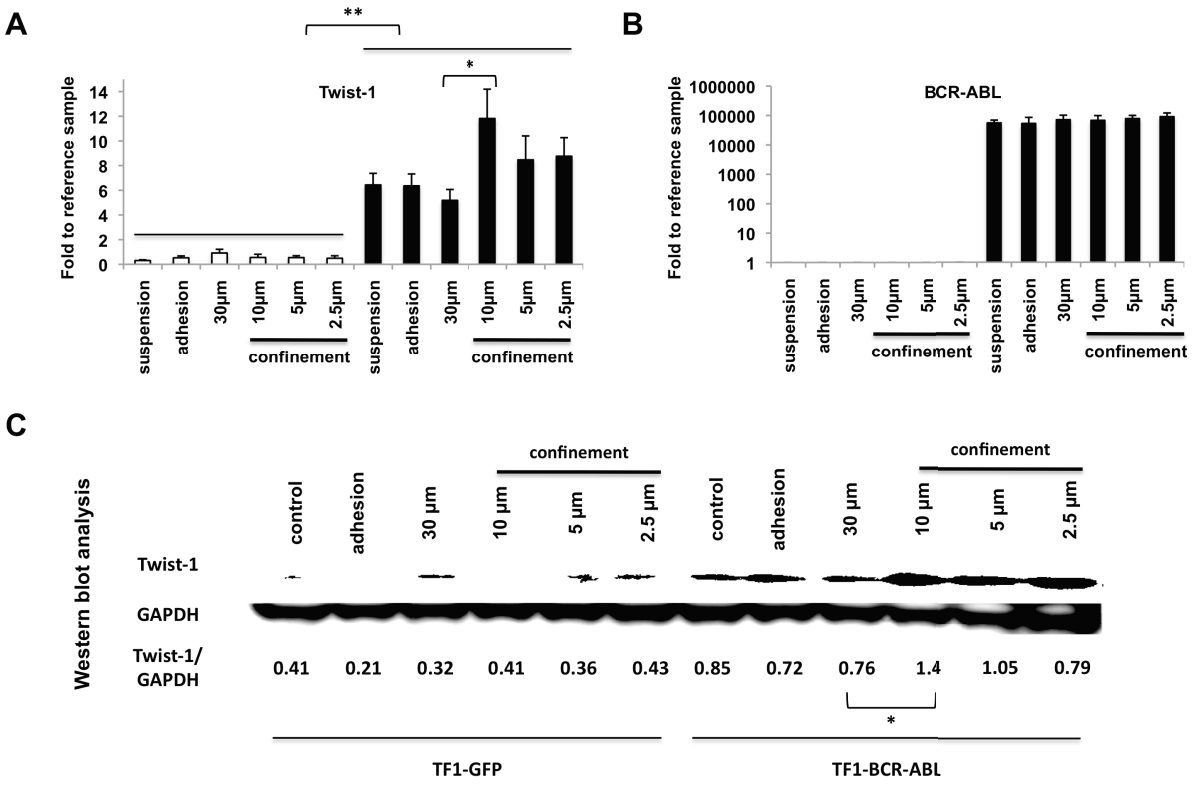

Figure 57 : Effect of mechanical stress on Twist-1 and BCR-ABL expression. (A) *Twist-1* and (B) *Bcr-Abl* genes expression was assessed by qPCR in wild-type (open bars) and BCR-ABL-transduced (closed bars) confined cells. Results are presented as the mean value ± SEM of n=10 experiments. (C) Twist-1 and GAPDH (used as loading control) protein analysis by Western blot in TF1-GFP and TF1-BCR-ABL cell extracts. Results are presented as the mean value ± SEM of n=4 experiments. *p<0.05; **p<0.005 indicate differences between two conditions.

4. Cell confinement impact on TF1-BCR-ABL cells proliferation is maintained upon Imatinib treatment

Twist-1 expression has previously been described by our group for its involvement in CML cells resistance to Imatinib treatment.[445] Thus, we asked whether Twist-1 induction by mechanical signals could alter the response of TF1-BCR-ABL cells to Imatinib treatment. We

then confined TF1-BCR-ABL cells in presence of 1µM Imatinib. After 3 days, cell proliferation was assessed using trypan blue staining and cell counting, demonstrating a strong inhibition of cell proliferation in response to Imatinib treatment (Figure 58A). While adhesion to fibronectin and 30 µm control slide had no significant impact on cell proliferation under Imatinib treatment, cell confinement impact on TF1-BCR-ABL cells was maintained under Imatinib treatment. Indeed, 10 µm confinement increased TF1-BCR-ABL cells proliferation, while 5 µm and 2.5 µm confinement induced a decrease in cell proliferation in a force-dependent manner. Thus, whereas cell confinement has no specific effect on Imatinib resistance despite an increased Twist-1 expression, it is still able to control cell proliferation under Imatinib treatment. Force-dependent modulation of TF1-BCR-ABL cells proliferation under Imatinib treatment was further correlated by a decrease in the percentage of Ki67-expressing cells (Figure 58B). The impact of cell confinement on CML cells proliferation under Imatinib treatment was also confirmed in KCL22 CML cells (data not shown). To assess the impact of mechanical stress on resistant $CD34^+$ CML cells, we cultured TF1-BCR-ABL cells for 2 weeks in presence of 1µM Imatinib to select resistant cells. These cells were then confined for 3 days in presence or not of 1µM Imatinib before evaluation of cell proliferation (Figure 58C). We observed similar effects of mechanical stress on proliferation of Imatinib-treated or untreated TF1-BCR-ABL cells. Indeed, in both cases, a slight confinement (10 µm) led to an increase of cell proliferation that was then lost after increasing the confinement (5 µm and 2.5 µm).

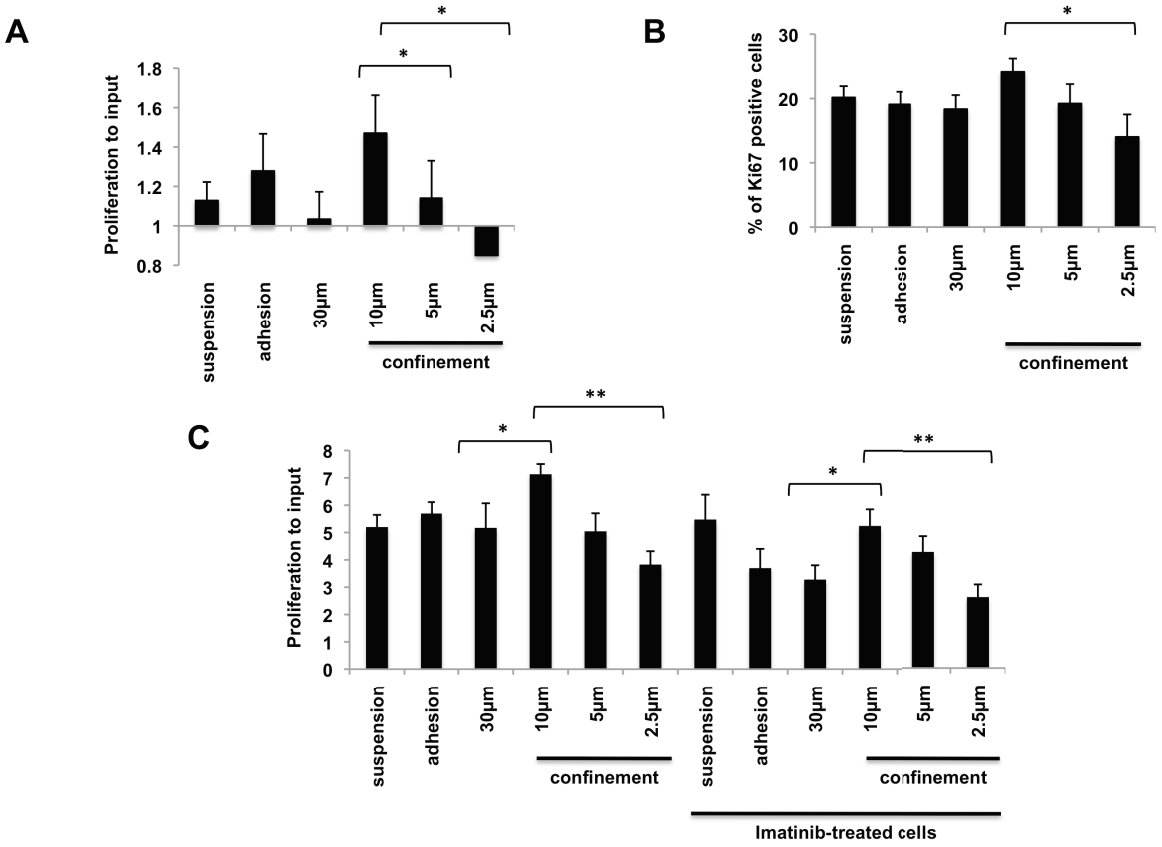

Figure 58 : Effect of mechanical stress on CML response to Imatinib. (A) Cell proliferation was evaluated in BCR-ABL-transduced confined TF1 cells after treatment with 1µM Imatinib using trypan blue staining and cell counting. Results are expressed as the proliferation ratio that is the ratio of cell counts to the number of input cells. (B) Phenotypic analysis of the proliferation marker Ki67 was then performed using flow cytometry on a Facscalibur® cell analyzer (Becton Dickinson) and gated for viable cells. (C) TF1-BCR-ABL cells were cultured 2 weeks in presence of 1µM Imatinib to select resistant cells and were then confined for 3 days ± 1µM Imatinib. Cell proliferation was then evaluated using trypan blue staining and cell counting. Results are expressed as the proliferation ratio that is the ratio of cell counts to the number of input cells and are presented as the mean value ± SEM of n=4 experiments. *p<0.05; **p<0.005 indicate differences between two conditions.

III. Discussion

The ability of cells to respond to changes in their physical environments is crucial in the development and maintenance of tissues that are exposed to varying mechanical stress.[279] Changes in tissue stiffness,[306] cell stiffness[286] and tumor growth due to proliferating cells[316] combine to affect the physical environment of both normal and tumor cells.[418] This altered physical environment can in turn modulate the fate of cancer cells through mechano-transduction signaling. Indeed, for a tumor to grow in a confined space defined by the surrounding tissue, it must overcome the resulting compressive forces.[316] However, very few studies have directly investigated the influence of mechanical stress on cancer cells biology.

Here we have reported that a mechanical stimulus applied to immature leukemic cells adhered to a fibronectin matrix control the proliferation of these cells. This ability appears to be unique to leukemic cells, as mechanical stimulus did not affect normal cells. Indeed, BCR-ABL transduction was sufficient to trigger an altered proliferative response of $CD34^+$ TF1 cells under mechanical stress. However, mechanical forces displayed a dual role in TF1-BCR-ABL cells proliferation control, as cell proliferation was increased under slight confinement and decreased under higher confinements. Finally, we demonstrated that Twist-1 expression was specifically increased in response to mechanical stress in $BCR\text{-}ABL^+$ cells. Interestingly, Twist-1 is involved in cell-cycle regulation[525] and was recently demonstrated to control quiescence[526] and self-renewal[526] capabilities of hematopoietic stem cells. Thus, Twist-1 modulation could be involved in the control of $CD34^+$ TF1-BCR-ABL cells proliferation under mechanical stress. These preliminary results demonstrate that BCR-ABL expression is required for TF1 cells to respond to mechanical signals. Strikingly, these results are in line with a recent study demonstrating that Twist-1 is expressed in response to transient compression in APC deficient colon tissue explants, but not in wild-type colon explants.[315] This suggests a potential role for Twist-1 as a common mediator of mechanical signals transduction in tumor cells. Thus, physical factors could be involved in existing cancer cells expansion and may potentiate cancer progression. This work provides unique insight into how physical determinants can influence immature leukemic cell fate that could help to explain how dividing cancer cells can take advantage of the constraints exerted by their microenvironment to promote tumor growth. These results emphasize the importance of a multidisciplinary approach in the study of immature cancer cells involving the coalescence of many disciplines, including cell and molecular biology and biomechanical engineering.

Discussion

Cancer, cancer stem cells and microenvironment

Cancer remains one of the leading causes of mortality worldwide. Clinically, the CSC discovery predicts that if the destruction of tumor bulk induces remission, only the eradication of CSC can lead to a cure.[85] Just like normal SCs,[50] CSCs are located in a peculiar microenvironment. However, the response of CSCs to the signals provided by their microenvironment can be totally altered, as CSCs are able to subvert the tumor niche to survive to promote their expansion and/or survival upon treatment.[527] Thus, it is necessary to understand the key processes occurring within the CSC niche to identify potential therapeutic targets that can serve as the basis for development of more effective treatments, targeting CSC within their microenvironment. What is more, targeting CSCs in their microenvironment must be done without damaging normal SC. Thus, it is essential to understand the specific alterations that occur in the formation of both CSCs and their altered microenvironment.

The actual dogma for cancer formation is that it is due to the accumulation of genetic alterations in a more or less immature cell. This is strongly supported by the CML model, where BCR-ABL is generally accepted as the first and sufficient alteration to promote leukemic formation, without any (or few) identified risk factors. The tumor formation can then lead to the alteration of the CSCs' microenvironment, which will in turn participate to the tumor phenotype.[486] In the course of the disease, cancer cells can then acquire more and more secondary alterations, making them even more difficult to eliminate. This is particularly important in the CML context where BCR-ABL induces genomic instability by inhibiting effective DNA repair. Thus, it is essential to detect and to understand early phase of cancer initiation (chronic phase of the disease for CML) where we still have a chance to overtake the disease.

However, alteration of the stem cell microenvironment has also been demonstrated as the first initiating event leading to tumor formation. The best example is referred as donor cell leukemia (DCL).[528] Relapse of acute leukemia following hematopoietic stem cell transplantation usually represents return of an original disease clone, having evaded eradication by pretransplant chemotherapy or radiotherapy. However, in almost 5% of all post-transplant leukemia relapses, acute leukemia can develop de novo in engrafted cells of donor origin without actual development of leukemia in the donor. While mechanisms of donor cell transformation are still unclear, it has been suggested that alterations of the bone marrow microenvironment due to myeloablative treatment could be responsible of donor cells leukemic transformation. In addition, recent work in our group suggests that the environment could be the first initiating event leading to tumor formation.[425] Indeed, it was demonstrated that alteration of the tumor microenvironment by environmental pollutants leads to tumor initiation in breast cancer. If we look further into this, it means that the first event in cancer

formation could be the alteration of SCs microenvironment, thus favoring the transformation of SCs into CSCs and leading to cancer formation. Coming back to CML, such alteration of the BM microenvironment could possibly be needed to induce BCR-ABL formation in a HSC. The existence of such a mechanism could explain why BCR-ABL oncoprotein is sometimes detected in human blood cells, without the development of CML all along the lifetime of these individuals.[529] While no proof of such mechanism has been found so far, the formation of a permissive microenvironment could then lead to CML appearance upon BCR-ABL formation, or could even favor t(9;22) chromosomal translocation.

Actually, more and more evidences demonstrate that incorrect signals from the microenvironment can lead to destabilization of tissue homeostasis and initiation and promotion of normal cells to malignancy.[84,485,511,530–532] However, in some cases, the microenvironment could also contribute to restrain cancer progression. Indeed, it has been demonstrated that the microenvironment can provide crucial signals to maintain tissue architecture inhibit cell growth and suppress or revert the malignant phenotype.[533]

CML : a model for stem cell transformation and leukemic stem cell resistance

In this context, CML was demonstrated to be a clonal disease[87,89] that origins from to the formation of the BCR-ABL oncogene in a single multipotent HSC.[86] This unique genetic abnormality allowed the researchers to develop targeted therapies blocking BCR-ABL tyrosine kinase activity. Imatinib, the first tyrosine kinase inhibitor (TKI) developed, has proved to be very efficient to induce the remission of a majority of CML patients.[131] However, none of TKIs available to date seem to eradicate undifferentiated BCR-ABL$^+$ cells that may serve as a reservoir for additional oncogenic events leading to disease progression and requiring continued treatment.[92,144,181] Therefore, CML represents a unique model to study LSC biology and to elucidate some of the mechanisms of therapeutic resistance. In CML, different mechanisms involved in resistance to TKIs have already been identified.[488] However, in some cases, the resistance mechanisms are still unknown, and could be explained by the existence of LSCs, that could survive to drug treatment due to their intrinsic specific properties and to their interactions within their microenvironment. Indeed, recent studies have demonstrated that LSC are particularly resistant to TKI treatments because of specific intrinsic properties, but also due to their protection by the leukemic niche.[154] Despite the success of TKIs, the wide range of resistance mechanisms observed in CML cells (detailed in chapter 2) is striking. Thus, different alterations (of LSCs but also of their microenvironment) are responsible of TKIs resistance mechanisms, in line with our results demonstrating that the decrease of *Smad6* expression is not present in all resistant CML

patients. Hence, it is necessary to understand the different mechanisms involved in CML resistance, but also to develop markers and new treatments to detect and overcome resistance. In particular, understanding BCR-ABL-independent resistance mechanisms could be useful as a model to understand resistance mechanisms in other types of cancer. At this time, more and more molecules are developed to target specific alterations in cancers. From here, the concept of personalized medicine will probably allow to develop targeted therapies adapted to the molecular alterations specific of each cancer, in each patient. For example, in breast cancer, treatment decision is based on the detection of specific alterations such as estrogen receptors over-expression or Her2 expression.

BMP pathway, CML and resistance

We demonstrated the existence of molecular and functional alterations in the BMP pathway in CML cells, as well as a significant increase in the availability of soluble BMP2 and BMP4 in the BM of CP-CML patients at diagnosis.[486] These alterations, and particularly the increased expression of BMPRIb in immature CML cells, are involved in the chronic phase of the disease through their role in the survival of LSCs as well as in the expansion of myeloid progenitors (Figure 59).[486] Soluble BMPs, naturally provided by the BM niche,[482] have been implicated in myelofibrotic processes.[420] In CML, changes in the SC niche such as secondary myelofibrosis are often correlated with disease progression.[419] Myelofibrosis is characterized by an increased production in collagens, proteins known to sequester BMPs,[534–536] and control the gradient of these molecules in the microenvironment. In addition, TKIs induce BMP2 expression by mesenchymal SCs.[421] Furthermore, BMP4 has been demonstrated to increase hematopoietic progenitor adhesion to the stroma and regulate HSC behavior.[399,412,476] Therefore, CML evolution and TKI treatment may contribute to a local increase in BMPs in the LSC niche, This also explains why both normal and leukemic SCs, as well as BM alterations observed in patients, are amplified throughout disease progression and may fuel a permanent and autonomous pool of leukemic progenitors.

While we demonstrated an overall increase in BMPRIb expression in $CD34^+$ CP-CML cells compared to healthy ones, not all $CD34^+$ CP-CML cells and/or samples display an over-expression of BMPRIb.[486] With regard to *low-BMPRIb* expressing samples, it could simply mean that a small immature CML cell sub-population with a high expression of BMPRIb is responsible of the amplification of myeloid leukemic progenitors and LSCs. To answer that, it would be necessary to sort low- and high-BMPRIb expressing immature cells from a unique CP-CML patient and to assess their functional response to soluble BMPs. This is supported by our results demonstrating that BMP response is particularly strong in high-BMPRIb expressing samples.[486] However, BCR-ABL expression alone has been demonstrated to be

sufficient to induce leukemic phenotype in both human cells and mice models.[537,538] Thus, BCR-ABL-mediated BMPRIb over-expression could simply constitute an additional transforming element cooperating with BCR-ABL and participating to the leukemic phenotype. As we have no mean to know precisely when the t(9;22) translocation leading to BCR-ABL formation occurred in patients, we cannot discriminate BMPRIb expression depending on the time after transformation, that could help us to understand the differences observed between patients. However, when separating CP-CML patient samples based on their Sokal score, we found no differences in BMPRIb expression between high- and low-Sokal CP-CML samples.[486] Thus, it would be interesting to design of mouse model over-expressing BMPRIb in HSC to observe if a CML-like disease appears. BCR-ABL inhibition in BMPRIb over-expressing CML cells could also be performed, but would entail more bias, such as BCR-ABL-induced secondary alterations, or difficulties to totally abolish its expression.

Furthermore, considering the crucial role for BMP alterations observed in primary CP-CML cells *in-vitro*,[486] it would be very interesting to observe the functional consequences of such alterations *in-vivo*, to also consider the effects on the BM microenvironment. However, while mice models are currently used as a standard in cell biology, several studies have demonstrated that BMP molecules (and others) can have totally different effects on human or mice cells.[389,390] This is probably one of the reasons why some molecules that have proved to be efficient in mice fail to reproduce their activities when tested in human.[539] Thus, there would be no advantage of such a strategy to further assess the effects of BMPs on BM microenvironment. However, recent progress in sciences may allow circumventing these difficulties. Indeed, several groups are working on humanized mice, that is to say mice expressing specific human proteins, allowing them to ascertain that the signaling pathways studied in mice will be the same (or at least close) as in humans.[540]

Another problem that is of importance to study the functional effect of biological molecules and that is recurrent in cell biology is the concentrations used. Indeed, it is very difficult to precisely determine the concentration of such molecules in the human body, particularly in specific microenvironments, where soluble molecules can also be trapped by different ECM proteins, soluble competitors, or can be produced in autocrine or paracrine manners thus affecting their diffusion and their effects on neighboring cells. Furthermore, most studies use very high concentrations of such molecules for *in-vitro* assays, probably to make sure to observe the functional effects of the molecules studied. However, it has been demonstrated that the biological effects of some molecules can be totally different depending on the concentration used, as it is the case for BMPs.[388,409] Thus, it would be very interesting to test different concentrations of BMPs to observe possible differences in term of biological

impact in the CML context.

In addition, two transcriptome studies were recently published, demonstrating alterations of the BMP signaling pathway in CML LSC. In a first study, a genome-wide analysis comparing transcriptomes of healthy and CP-CML $CD34^+$, $CD38^-$, $ALDH^{high}$ immature cells was performed. Interestingly, an alteration of both TGF-β and BMP signaling pathways elements was observed in immature CP-CML cells compared to healthy ones. Particularly, *BMP2, BMP9* and *TGF-β1* gene expression was found to be down-regulated in immature CP-CML cells, while the expression of *SMAD7*, an inhibitor of the TGF-β signaling pathway, was highly activated.[541] This suggests a possible impairment of autocrine BMP2 and BMP9 production by immature CP-CML cells, while both TGF-β signaling and TGF-β1 autocrine production could be impaired. In line with this study, our results demonstrated a slight but non-significant decrease of *BMP2* expression in $CD34^+$ CP-CML cells compared to healthy ones.[486] However, in this study, the expression of these molecules was not assessed in more mature compartments, as well as the functional consequences of these molecular alterations. In addition, no validation of the protein expression levels was performed to confirm these results. Finally, another transcriptome study confirmed *BMP2* and *BMP4* down-regulation in CP-CML LSCs ($CD90^+$, $CD34^+$, $CD38^-$, Lin^-), whereas *BMP7* and *BMP10* were found to be up-regulated.[542] Although we focused our analysis on BMP2 and BMP4, these changes could sign an altered autocrine signaling in CP-CML LSCs. This study also confirmed *BMPRIb* over-expression in CP-CML LSCs and demonstrated that its expression was then progressively lost over the course of disease progression. This result confirms our description of *BMPRIb* increase as an early event in CML course, but is not in line with the result we obtained analyzing *BMPRIb* expression in 4 CML patients in advanced phase at diagnosis. Thus, while *BMPRIb* over-expression in CP-CML LSCs is validated, it is difficult to status for *BMPRIb* expression in advanced CML phases as the two studies were performed on few patient samples (4 acute phase and 2 blast crisis samples for the last study).

We also demonstrated a significant increase in the availability of soluble BMP2 and BMP4 in the BM of resistant CML patients compared to CML patients in remission under TKI treatment in part 2 of the results. We further showed that the BMP pathway is altered in cells from resistant CML patients compared to sensitive patients, and that some alterations seems to be specific of immature $CD34^+$ compartment. Indeed, we found that most of resistant CML patients display a decreased expression of Smad6, the expression of which seems to be involved in CML cells resistance to Imatinib treatment. Preliminary results suggest that loss of *Smad6* expression could lead to the increased *Twist-1* expression observed in immature cells from resistant CML patients, thus leading to Imatinib resistance (Figure 59). It would be interesting to see if different elements of the BMP pathway could also be involved in Twist1

expression control, and more interestingly, if this process is also true in more immature cells such as $CD34^+$ CML cells or TF1-BCR-ABL cells. Indeed, these results were obtained with mature $CD34^-$ KCL22 cells (with sensitive and resistant clones to TKI treatment). However, during my PhD, part of our team developed Imatinib-sensitive and Imatinib-resistant clones from immature $CD34^+$ TF1-BCR-ABL cells, which would constitute a much better model to study LSC-mediated resistance to Imatinib treatment. As described in chapter 4, BMPs induce *Smad6* expression, thus providing a ligand-induced negative-feedback loop for Smad signaling. We demonstrated that BMP signaling is involved in immature leukemic cells expansion. Thus, an inhibition of this negative-feedback loop could be responsible of the decrease of *Smad6* expression in immature CML cells even with high levels of soluble BMPs in the bone marrow microenvironment. This is supported by our results on KCL22 resistant cells that were unable to increase *Smad6* expression under BMP4 treatment. In addition, BMP2/4 treatment increased KCL22-resistant cells survival upon Imatinib treatment, while this was not the case for KCL22-sensitive cells. It is generally thought that Smad6 competitively interfere with the binding of Smad1/5/8 to type I receptors, thus preventing their phosphorylation and that it interferes with the BMP induced formation of the heteromeric Smad1–Smad4 complex. However, other studies demonstrated that Smad6 is also able to physically interact with Hoxc-8 and histone deacetylases (HDACs) in cell nucleus to repress BMP-induced gene transcription, such as *Id1*.[543] While the precise mechanism of how Smad6 acts as a transcriptional co-repressor remains to be elucidated, it would be interesting to perform immunofluorescence staining of immature CML cells to understand the localization and the possible action of Smad6 in BMP pathway control. In addition, Smad6 expression has been reported to be decreased upon mechanical stretch in rat mesangial cells.[375] We observed that loss of Smad6 expression induced Imatinib resistance in KCL22 cells. Thus, mechanical signals could lead to Imatinib resistance upon mechanical stress by inhibiting Smad6 expression. However, we found no difference in Smad6 expression in both TF1-GFP and TF1-BCR-ABL cells upon mechanical stress to support this hypothesis.

The mechanism by which IFN-α mediates its anti-leukemic effects in CML cells is still debated. Interestingly, the p38/MAPK pathway is activated by IFN-α in BCR-ABL-expressing cells and appears to play a key role in the generation of the growth inhibitory effects of IFN-α in CML cells.[544] However, IFN-α resistant CML cells failed to activate p38/MAPK signaling in response to IFN-α treatment. In addition, p38/MAPK axis has been identified as a key element in the non-canonical BMP signaling pathway.[343] Thus, an alteration of the BMP signaling pathway in CML cells could impair p38/MAPK activation eventually leading to cell resistance upon drug treatment.

While BMP signaling pathway is part of the TGF-β super-family signaling pathway, it

appears that these pathways bear different functional consequences in immature CP-CML cells. Indeed, while we demonstrated that alterations of the BMP pathway are involved in the expansion of immature CP-CML cells,[486] it has been shown that BCR-ABL transduction in TF1 cell line enhances their responsiveness to the TGF-β growth inhibitory activity.[443] Hence, while BMP signaling pathway seems to be involved in CP-CML phenotype, TGF-β signaling pathway could be involved in the quiescence and persistence of immature CML cells, maybe affecting their survival under TKIs treatment. These results illustrate the dual activity of BMP and TGF-β signaling and confirm the interest for recent mathematical models of CML that propose to combine Imatinib to a cell-cycle stimulant to eliminate quiescent and resistant CML cells.[545]

Osteoblastic niche, vascular niche and CML

Recent studies have put into light the existence and structurally and functionally different hematopoietic niches, called osteoblastic and vascular niches.[546] The composition of these two niches is different, and so are the interactions between HSCs and microenvironmental cells. However, while different functional consequences have been observed, the causes explaining them are not fully understood. For example, while this has not been illustrated yet, the mechanical properties of these niches may be different because of the different cell types constituting them. Thus, this could lead to the transduction of different mechanical signals inside HSCs possibly involved in the control of their fate. Moreover, the different cell types present in the niches probably produce different types or different quantities of soluble molecules. Interestingly, we demonstrated that BMP4 is mainly produced by sinusoid endothelial cells in CP-CML BM environments,[486] highlighting the crucial role of the vascular niche in CP-CML immature cells expansion. So far, the respective roles of osteoblastic and vascular niches in CML have not been particularly studied. Thus, the different components of the BM microenvironment could have different functional effects on immature LSCs, as this is the case for healthy HSCs. As the BMP pathway has been described as mechano-sensitive,[243] the differences in term of BMP production or signaling could be further induced by differences of mechanical signals. Indeed, as part of the bone marrow microvasculature, endothelial cells are subject to important mechanical stress induced by blood circulation. However, we did not observe any difference in *BMP2* or *BMP4* expression by confining BMEC-1 microvascular endothelial cells (data not shown).

BCR-ABL alters cell stiffness and cell response to mechanical signals

We demonstrated that BCR-ABL expression alone is sufficient to induce an increase of cell stiffness in $CD34^+$ CP-CML cells (Figure 59). Interestingly, differences in cell stiffness

have also been observed in many types of cancers including leukemia. While most solid tumor cells studied so far seem to be softer compared to healthy cells,[286,287,293,294] our results are in line with the differences of stiffness observed in other hematologic malignancies. Indeed, results from ALL and CLL cells analysis also found an increase of leukemic cell stiffness.[296,299,300] However, no common molecular mechanism has been identified so far that could explain the similarities between hematologic malignancies or the discrepancies with solid tumor cell stiffness alterations. Nevertheless, mechanical alterations were almost always correlated with alterations of cell cytoskeleton, often involving different degrees of F-actin polymerization. Interestingly, in transformed cells, BCR-ABL has been demonstrated to bind actin filaments (F-actin),[508] one of the major determinants of cell mechanical behavior,[509] and to induce its redistribution into punctate, juxtanuclear aggregates.[108] By performing immunofluorescence experiments, we were able to confirm these results in immature TF1 cells after BCR-ABL transduction. Considering the crucial role of F-actin in cell stiffness, the reorganization of actin cytoskeleton induced by BCR-ABL is probably involved in the differences of cell stiffness observed by AFM. Moreover, we also demonstrated that adhesion to fibronectin led to the stiffening of TF1 cells as a consequence of F-actin polymerization. Interestingly, adhesion to fibronectin increased the impact of BCR-ABL expression on TF1 cell stiffness and was correlated with a strong co-localization between F-actin and BCR-ABL as observed by immunofluorescence experiments. Furthermore, understanding the biological impact of these intrinsic cell mechanical alterations on cell functional properties has proved quite challenging. While we can easily assess gene and protein function by modulating their expression with modern molecular biology techniques, modulating cell stiffness is more difficult. Indeed, while we know how to modulate cell stiffness by acting on many components of the cytoskeleton, we cannot exclude that these alterations will also affect other cellular mechanisms such as cell division or vesicles transport since cytoskeleton is involved in many critical cellular processes. For example, a combination of different molecules, such as inhibitors of actin polymerization can be used to impair F-actin formation and to soften the cells. Actin over-expression by viral vector could then be used to perform the reverse experiment in order to stiffen the cells. However, F-actin is also involved in the regulation of numerous cell functions, probably not involving cell stiffness.

Changes in tissue stiffness,[306] cell stiffness[286,296,298] and tumor growth due to proliferating cells[316] combine to affect the physical environment of both normal and tumor cells. For example, the stroma surrounding a tumor is generally enriched in both type I collagen and fibronectin,[307] creating a denser and mechanically rigid tissue compared to normal tissue.[306] Moreover, uncontrolled growth in a confined space generates mechanical

compressive stress within tumors (Figure 59).[316] In addition, an increasing amount of studies demonstrate that cancer cells possess altered intrinsic mechanical properties compared to healthy cells.[286,296,298] Thus, tumor tissue displays different mechanical properties and will produce altered mechanical signals compared to healthy tissue. Considering the crucial role of mechanical signals on cell and stem cell fate control,[276,547] altered mechanical signals and/or cell mechanical properties could also impact the biology of cancer stem cells. To confirm the occurrence of compressive stress *in-vivo*, it could be interesting to compare cell deformation in bone marrow biopsies (keeping the bone marrow architecture) from healthy donors and CML patients, by measuring cell size and shape.

Our preliminary results also demonstrate that BCR-ABL expression is required for TF1 cells to respond to mechanical signals. This could sign BCR-ABL's ability to alter mechano-transduction signaling pathways in these cells. As BCR-ABL lead to an alteration of F-actin cytoskeleton in CML cells,[108] alterations in the tensional force generated by the actin/myosin apparatus may play a pivotal role in tumor cells functions. To analyze the role of BCR-ABL/F-actin binding in mechanical signals transduction, it would be necessary to design *Bcr-Abl* mutants with impaired binding ability to F-actin cytoskeleton. This strategy could also be combined to AFM to assess the role of BCR-ABL/F-actin binding in alteration of immature cell stiffness.

Only a few clues on hematopoiesis control by mechanical signals have been identified so far.[46,453,455,548,549] However, the crucial role for mechanical forces in hematopoiesis is perfectly illustrated by the growth of colonies in the CFC assay performed in semi-solid culture conditions using methylcellulose. Indeed, such terminal differentiation of hematopoietic progenitor cells seems to depend on physical signals provided by culture in a three-dimensional gel. This crucial influence of mechanical stress on cell functional properties put into light the need to develop and to use three-dimensional assays reproducing tissue organization and mechanical interactions to understand cell fate in a more physiological environment. In this study, using a unique system allowing us to confine a population of hematopoietic cells,[273,458–460] we report that a mechanical stimulus applied to immature leukemic cells adhered to a fibronectin matrix control the proliferation of these cells. This ability appears to be unique to leukemic cells, as mechanical stimulus did not affect normal cells. Indeed, BCR-ABL transduction was sufficient to trigger an altered proliferative response of $CD34^+$ TF1 cells under mechanical stress. However, mechanical forces displayed a dual role in TF1-BCR-ABL cells proliferation control, as cell proliferation was increased under slight confinement and decreased under higher confinements. Finally, we demonstrated that Twist-1 expression was specifically increased in response to mechanical stress in $BCR\text{-}ABL^+$ cells. Interestingly, Twist-1 is involved in cell-cycle

regulation[525] and was recently demonstrated to control quiescence[526] and self-renewal[526] capabilities of hematopoietic stem cells. Thus, Twist-1 modulation could be involved in the control of $CD34^+$ TF1-BCR-ABL cells proliferation under mechanical stress. To assess this hypothesis, it would be interesting to modulate Twist-1 expression (plasmid vectors and shRNA) in TF1-BCR-ABL cells and evaluate the impact on cell proliferation. It would also be necessary to inhibit Twist-1 expression in TF1-BCR-ABL cells during cell-confinement to observe if cell confinement is still able to control TF1-BCR-ABL proliferative capabilities in these conditions. In addition, it would be interesting to perform LTC-IC assays after primary $CD34^+$ CML cells confinement to test the impact of mechanical stress on CML LSCs self-renewal capabilities, that have been linked to Twist-1 expression. Strikingly, these results are in line with a recent study demonstrating that Twist-1 is expressed in response to transient compression in APC deficient colon tissue explants, but not in wild-type colon explants.[315] This suggests a potential role for Twist-1 as a common mediator of mechanical signals transduction in tumor cells. Thus, physical factors could be involved in existing cancer cells expansion and may potentiate cancer progression. Interestingly, the impact of mechanical signals on TF1-BCR-ABL cell proliferation was conserved under Imatinib treatment, but with lower rates of proliferation. Twist-1 expression has been involved in TKIs resistance, and is increased in TF1-BCR-ABL cells under confinement. Yet, Twist-1 induction by mechanical signals was not sufficient to increase the resistance of TF1-BCR-ABL cells to TKI treatment in our experiments.

While we observed no impact on BMP pathway elements gene expression in CD34+ TF1-BCR-ABL cells, preliminary results demonstrate that mechanical signals could be involved in BMP pathway regulation through activation of Smad1/5/8 effectors phosphorylation (data not shown). Indeed, such mechanism has already been described to either activate or inhibit cell response to soluble BMPs.[243] In addition, it would be interesting to assess BMP inhibitor genes expression, also controlled by mechanical stress. Thus, while mechanical stress had no impact on BMP2 or BMP4 gene expression (in TF1-GFP, TF1-BCR-ABL as well as in stromal cells, data not shown), it could still be involved in cell response to soluble BMPs present in the BM microenvironment. Interestingly, Twist-1 is able to bind to the Runx1 Runt domain and decrease Runx1 transactivation activity.[526] Runx1 plays important roles in hematopoiesis, particularly during embryogenesis,[550] and is described as a downstream target of BMP signaling pathway. Thus, Twist-1 could act as a mediator to control BMP pathway signaling through regulation of BMP target genes under mechanical stress. Moreover, even if the system we used allows us to reproduce mechanical signals *in-vitro*,[458] it is not sufficient to reproduce 3-dimensional mechanical parameters such as capture and release of soluble molecules by ECM under mechanical stress.

Probing hematopoietic cells stiffness

So far, only a few groups have tried to measure hematopoietic cells mechanical properties. While micro-fluidic devices, micropipette aspiration or optical stretcher were sometime used, most studies were performed using AFM technology.[296,298–300] Moreover, because hematopoietic cells tend to slip from under the cantilever when performing AFM indentation, we have to immobilize these cells. Up to now, these different groups designed micro-fabricated wells or pillars to mechanically immobilize hematopoietic cells and to probe them. In our study, we combined the design of micro-fabricated wells to hematopoietic cell adhesion on fibronectin-coated cover-slips to immobilize them. This also allowed us to study the impact of cell adhesion to ECM on their stiffness.

We and others demonstrated that hematopoietic cells are very soft cells.[298] This is probably due, at least in part, to the low level of F-actin polymerization (compared to adherent cells), which is involved in cell stiffness control. Moreover, due to the size of their nucleus and to their low F-actin content, hematopoietic cells are generally round cells. Interestingly, as in liquid droplets, a round morphology tends to minimize the mechanical constraints at the cell surface.[551] Thus, such a round morphology could also be involved in hematopoietic cell softness. However, this is not true when hematopoietic cells become adherent to ECM proteins, such as fibronectin. Indeed, in that case, we observed different cell morphologies together with an increased F-actin polymerization consistent with the increased stiffness observed in adherent hematopoietic cells.

Finally, we also observed that primary $CD34^+$ cells are much more stiffer as compared to $CD34^+$ TF1 cells. Thus, we have to be careful when interpreting AFM studies, some of which comparing stiffness results obtained from both primary cells and cell lines.

Comparing healthy and leukemic cells' mechanical properties can prove very difficult for different reasons. First, and particularly for leukemic cells, it is difficult to assess the ratio of healthy to leukemic cells that are both present in blood samples of patients. That is why the CML model is very interesting, because it is easy to control the percentage of $BCR\text{-}ABL^+$ cells, that is to say the leukemic cells content. The other advantage of this model is that the first transforming event being identified, it can be reproduced in *in-vitro* studies to confirm the role of BCR-ABL in cell stiffness differences.

Moreover, hematopoietic cells are not a homogeneous population, as many cell types with different properties co-exist in this tissue. Thus, these cells can display different intrinsic structures leading to different mechanical properties. Indeed, a recent study demonstrated that myeloid cells were 18 times stiffer than lymphoid cells and six times stiffer than neutrophils on average.[298] However, these results have to be confirmed as they were obtained comparing cell lines to primary cells (HL60 cell line, Jurkat cell line and primary

neutrophils respectively). Indeed, we demonstrated that primary cells were much more stiffer as compared to the TF1 cell line. To circumvent this difficulty, we selected $CD34^+$ cells from both healthy donors and CP-CML patients, allowing us to compare a more homogeneous cell population. This is very important as up to now, mechanical analysis on hematopoietic cells were performed on heterogeneous leukocytes populations.[296,299–301] But, CML being characterized by an expansion of myeloid progenitors, we cannot exclude that the results we observed are due to an increased proportion of myeloid progenitors among CP-CML $CD34^+$ cells. This would be in line with the increased stiffness observed in myeloid cells in the aforementioned study. Despite that, we were able to demonstrate that BCR-ABL expression alone was able to increase the cell stiffness of TF1 cells. Thus, this demonstrates that BCR-ABL is at least partly involved in the increased cell stiffness that we observed in primary $CD34^+$ CP-CML cells.

Pitfalls in cell mechanics studies

Interestingly, a recent study demonstrated the progressive alteration of cell mechanical properties after culture in a two-dimensional stiff environment. By combining AFM and optical stretching, it was evidenced that mesenchymal SC stiffness dramatically increased during extended passaging on stiff substrata.[552] This cell stiffening was correlated with an increased polymerization of actin stress fibers, and was only observable when the cells were adherent. Indeed, this effect was lost with cell detachment at each passage, and was anew observed after each cell reattachment to the substrata. This study further supports the impact of environment on cell stiffness, and demonstrates that substrata stiffness can have a durable impact on cell stiffness and/or select a population of stiffer cells probably more suited to persist in these conditions. Thus, when comparing cell mechanical properties, it is important to consider the potential impact of cell culture and to minimize it by culturing the cells for a same and short amount of time. What is more, a study recently evidenced that cell confluence could impact their stiffness, single adherent cells being stiffer as compared to confluent cells cultured in monolayer.[553] Interestingly, other factors such as cell apoptosis,[301] aging[554] and nutrients consumption[555] were also demonstrated to affect cell stiffness. In addition, while it is not physiological for mesenchymal cells to be in a suspended state, this study also demonstrated the stiffening of an adherent cell over tens of minutes after being released from substratum contact.[552] This was correlated with changes of cell surface topography as well as with a reduction of actin stress fibers. These results seem surprising at first, as reduction of F-actin is usually associated with decrease of cell stiffness. However, F-actin cortical reinforcement was also observed as membrane-cortex connections developed and strengthened. Thus, the shift from a stress fiber to a cortical mechanical response could

then explain the differences observed. Finally, lack of a unique and reliable analysis method is a severe hindrance to the comparison of these different AFM studies. The wavelet-based extraction method of the shear modulus introduced in our study encompasses other parameterization methods, allowing a direct scale matching of the analyzing wavelet with the local force-indentation response curvature.

In part 4 of the results, we used the cell-confining device developed by M. Piel (Institut Curie Paris) to apply mechanical forces on hematopoietic cells. Just as for AFM experiments, we had to immobilize hematopoietic cells to confine them, by allowing the cells to adhere onto fibronectin-coated wells. To allow the testing of multiple conditions in the same experiments, we designed modified standard polystyrene 24-well plates in which PDMS pillars fixed on the plate lid held the micro-structured slides (Figure 27). We used 14 mm diameter confinement glass slides (slightly smaller than the 15 mm wells) bearing PDMS micro-structures to confine adherent hematopoietic cells. However, about 15% of cells are found in the zone between the cover-slip and the side of the well, and are not confined during the experiments. Thus, this probably dampens the consequences of mechanical signals that we observe, as some of the cells are actually not confined. Another method that could be used to confine hematopoietic cells would be to perform CFC assays with different concentrations in methycellulose, thus creating a range of meshing that will confine cells during their expansion. This would be interesting to maintain cell confinement during the CFC assay (2 weeks), since the cell-confining system cannot be used in semi-solid medium. Indeed, in our experimental setup, the confinement was released after 3 days and the cells were then plated in methylcellulose (without additional confinement for 2 weeks).

Cell stiffness and leukostasis

In CML, BCR-ABL$^+$ cells of the myeloid lineage proliferate uncontrollably. Leukostasis, a poorly understood condition in which cells aggregate in the vasculature, is commonly observed in patients with myeloid hematologic malignancies such as AML, but is also observed during CML blast crisis.[556] Indeed, during CML blast crisis, decreased cell deformability is thought to influence vascular flow and contribute to vascular complications such as leukostasis.[557,558] As myeloid cells are stiffer compared to other hematopoietic cells,[298] the amplification of the myeloid compartment in CML could lead to an increase of stiff cell content, thus potentially leading to leukostasis. Moreover, we evidenced that BCR-ABL expression alone is sufficient to increase immature cell stiffness, and could thus potentially reduce the capability for these cells to circulate through blood vessels, thus leading to leukostasis. In addition, such a mechanism has already been identified in pediatric ALL, where cells extracted from ALL patients with leukostasis were stiffer compared to cells

obtained from ALL patients without leukostasis.[299] This condition often results in intracranial hemorrhage and respiratory failure that rapidly leads to death. Thus, better knowledge of biophysical changes in leukemic cells such as deformability is necessary to improve understanding of the disease.

Cell stiffness, microenvironment stiffness and BMP production in CML

Unlike most tissues, hematopoietic tissue is mainly composed of non-adherent cells that are organized differently compared to adherent cells. However, this doesn't mean that these cells have no physical interactions with each other. This is particularly true for immature hematopoietic cells that are adherent to their bone marrow stroma, composed of ECM but also of stromal cells and other hematopoietic cells. In CML, we demonstrated that BCR-ABL expression alone is sufficient to induce a stiffening of immature $CD34^+$ cells. Interestingly, an emerging concept that has been recently described hypothesizes that the cells stiffness will be dependent on their microenvironment stiffness. This concept, known as mechano-reciprocity imply that stiff cells will preferentially grow in stiff environments and vice-versa.[241,418] Indeed, it has been demonstrated that cells are capable to adapt their stiffness to fit with the rigidity of their environment.[552] However, this concept is still debated in cancer cells as most of the studies have demonstrated that cancer cells are softer compared to their normal counterparts,[286,290,294] while cancer microenvironment (and particularly the ECM) seems to acquire stiffer characteristics[306] over the course of the disease. So far, we cannot really conclude about the relevance of such concept in cancer processes, as most cancer cells stiffness studies were in fact metastatic cancer cell studies. Interestingly, the metastatic potential of cancer cells was correlated with a decrease of cell stiffness in several studies. Thus, cell softening could be necessary for metastatic processes, in which cells have to break through several tissues, to migrate through blood circulation and to colonize different (mechanical) environments. Furthermore, the observed differences between tissue stiffness (increased) and cell stiffness (decreased) in tumor context seem contradictory, probably because of the crucial role of the ECM that appear stiffer in different tumor types.[306,310,312] But, in CML, this concept could also means that the stiffening of immature cells induced by BCR-ABL could lead to a reciprocal alteration of their microenvironment's stiffness. However, the mechanisms controlling mechano-reciprocity between cells and their environment are still unknown.

Such mechanism could for example involve the over-production of BMPs molecules. Indeed, BMPs have been well described to control mesenchymal SC differentiation.[414] Thus, the increase amount of BMP2 and BMP4 that we observed in the bone marrow of CP-CML patients[486] could lead to mesenchymal SC differentiation into specific lineages, leading to the

alteration of the BM microenvironment due to the presence of a particular set of cells producing specific factors such as ECM proteins. This could eventually lead to the alteration of the BM microenvironment stiffness, with possible induction of myelofibrosis, a phenomenon often observed with disease progression.

This idea brings us back to the production of soluble BMPs in the bone marrow of CP-CML patients. While we demonstrated that BMPs are synthesized in abnormally high quantities by the BM microenvironment of CP-CML patients, we still don't know the exact mechanism that triggered this over-production.[486] One of the hypothesis that could explain this production is the increase of the mechanical stress in the tumor microenvironment. Indeed, BMPs are well-known mechano-sensors whose production can be controlled by mechanical forces applied to the cells.[243] In CML particularly, BCR-ABL formation leads to an uncontrolled proliferation of hematopoietic cells in their BM microenvironment, potentially leading to cell compaction within this defined environment. This increased cell confinement could lead to differential expression of mechano-sensitive genes such as *BMPs*, or *Twist-1* as demonstrated in part 4 of the results. Moreover, this abnormal cell compaction will probably confine hematopoietic cells, leukemic cells, but also stromal cells present in this environment, potentially also affecting their production of soluble BMPs. Another interesting question is the possible impact of BMPs production on cell stiffness.

While no proof of such a mechanism has been evidenced yet, cell stiffness control by soluble molecules cannot be excluded. For example, BMP2 is able to induce rapid actin cytoskeleton reorganization in mesenchymal SC,[559] thus probably leading to the alteration of their mechanical properties. In addition, BMPs are known controllers of SC differentiation both in mesenchymal[414] and hematopoietic[399,404] systems. Thus, BMP over-production in CP-CML BM niche could lead to the amplification of specific lineages with specific intrinsic mechanical properties, as it is the case for myeloid progenitors in CML.[298]

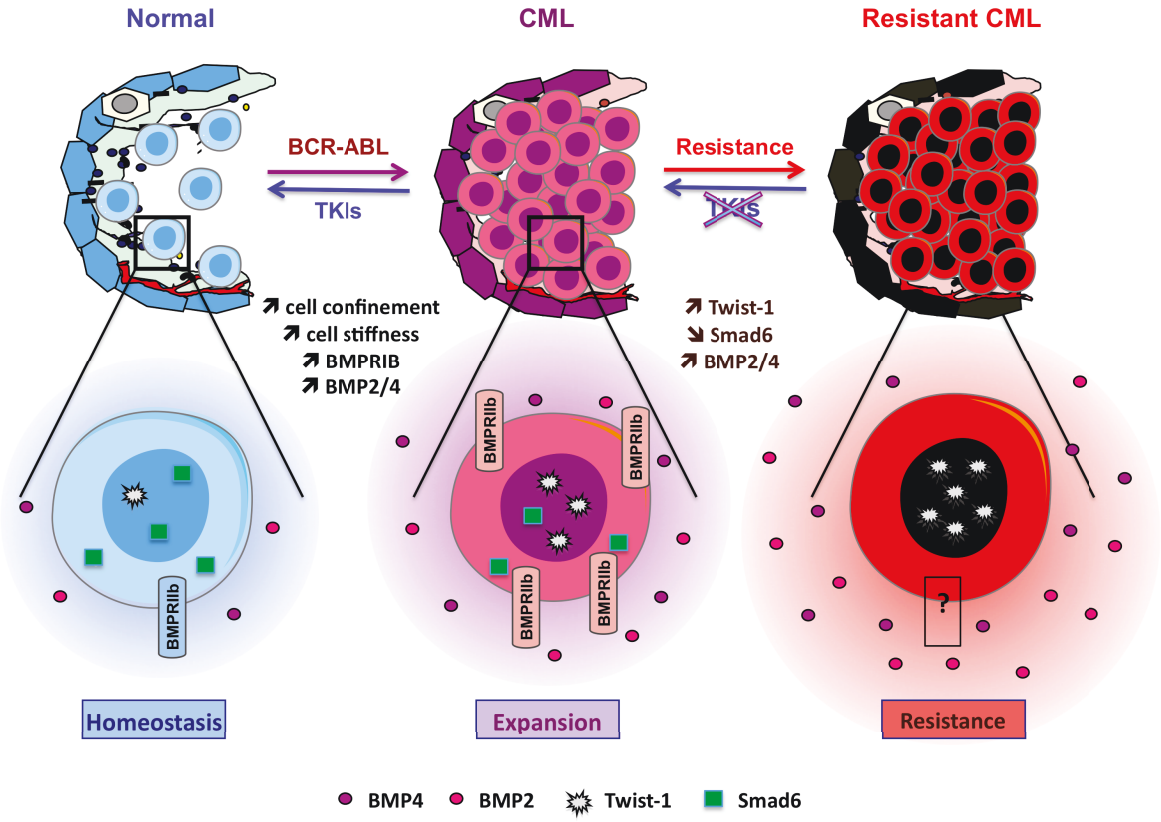

Figure 59 : Proposed model for BMP pathway and cell confinement effects on CD34$^+$ CML cells maintenance and resistance to TKIs. In the normal context, hematopoiesis is tightly controlled. However, in CML, BCR-ABL induces an increase of BMPRIb expression at the surface of immature CP-CML cells as well as the stiffening of these cells. Together with an increase in BMP2 and BMP4 levels in the bone marrow microenvironment, BMPRIb over-expression leads to the expansion of myeloid progenitors and LSCs. This potentially induces cell compression in the bone marrow, producing mechanical signals that promote Twist-1 expression. In CML patients resistant to TKIs treatment, Smad6 expression is lost, and could be responsible of the increased Twist-1 expression involved in TKI resistance. Finally, BMP2 and BMP4 levels seem even more elevated in the bone marrow of TKIs-resistant CML patients compared to TKIs-sensitive ones, and could also be involved in cell resistance to TKIs.

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Appendix

Oligonucleotide primers for human gene expression analysis by qRT-PCR

Gene	Sense primer	Antisense primer
TBP	CAC GAA CCA CGG CAC TGA TT	TTT TCT TGC TGC CAG TCT GGA C
BGUS	CGC CCT GCC TAT CTG TAT TC	TCC CCA CAG GGA GTG TGT AG
HPRT	TGA CCT TGA TTT ATT TTG CAT ACC	CGA GCA AGA CGT TCA GTC CT
BMP2	AGA CCT GTA TCG CAG GCA CT	CCT CCG TGG GGA TAG AAC TT
BMP4	CTT TAC CGG CTT CAG TCT GG	GGG ATG CTG CTG AGG TTA AA
BMPRIa	GAA AAA GTG GCG GTG AAA GT	TAG AGC TGA GTC CAG GAA CC
BMPRIb	GCC AGC TGG TTC AGA GAG AC	CAG GAC CCT GTC CCT TTG AT
BMPRII	TAG CAC CTG CTA TGG CCT TT	CTG AAT TGA GGG AGG AGT GG
Smad1	CGC GTT CCT TCT GAA AAT TG	TGC AAA AGG ACA GCA GAA GA
Smad4	TTT CCT TGC AAC GTT AGC TG	ATG CAC AAT GCT CAG ACA GG
Smad5	TCT GCT TGG GTT TGT TGT CA	GCA GCT GCT GGG AAT CTT AC
Smad6	CTG CAA CCC CTA CCA CTT CA	AGA ATT CAC CCG GAG CAG T
Smad7	TCC TGC TGT GCA AAG TGT TC	AAA TCC ATC GGG TAT CTG GA
Smad8	AAG TGT GCA TTA ACC CTT ACC A	AGG CTG AGC TGG GGG TTA T
Bambi	ATC GCC ACT CCA GCT ACA TC	GCA TCG AAT TTC ACC TTT GG
Foll	TCT GCC AGT TCA TGG AGG AC	CCC GTT GAA AAT CAT CCA CT
FLRG	GGG CTT CGT GAG CTC CAT	ACC AGG CGG TGT CAA TGT
Id1	GGT GCG CTG TCT GTC TGA G	TGT CGT AGA GCA GCA CGT TT
Id2	TAT TGT CAG CCT GCA TCA CC	AAT TCA GAA GCC TGC AAG GA
Id3	AAA TCC TAC AGC GCG TCA TC	AAG CTC CTT TTG TCG TTG GA
Runx1	TGG AGG AGG GAA AAG CTT CA	CCG ATG TCT CTT CGA GGT TCT C
Runx2	GTG GAC GAG GCA AGA GTT TC	TTC CCG AGG TCC ATC TAC TG
Runx3	CTT CAA GGT GGT GGC ATT G	GCT CAG CGG AGT TCT CG
Abl	TGG AGA TAA CAC TCT AAG CAT AAC TAA AGG T	GAT GTA GTT GCT TGG GAC CCA
Bcr-Abl	TCC GCT GAC CAT CAA TAA GGA	CAC TCA GAC CCT GAG GCT CAA
BMI	GAT ACT TAC GAT GCC CAG CA	GGA CCA TTC CTT CTC CAG GT
Twist-1	GGC TCA GCT ACG CCT TCT C	CCT TCT CTG GAA ACA ATG ACA TCT

BMP, Bone Morphogenetic Protein; BMPR, BMP receptor; Foll, Follistatine; TBP, Tata Box binding Protein; Id, Inhibitor of differentiation; Runx, Runt-related; Abl, Abelson; Bcr-Abl, Breakpoint cluster region-Abelson; Bambi, Bmp and activin membrane-bound inhibitor; BGUS, beta-glucuronidase; Smad, Small body size mothers against decapentaplegic; Twist-1, twist-related protein 1; FLRG, follistatin-related gene.

Scientific communications

Oral communications

Journée de recherche LMC (Bordeaux, 2012) : La voie des BMPs est altérée au niveau fonctionnel et moléculaire dans la LMC en phase chronique.

Journée des doctorants en physique de l'ENS (Lyon, 2013) : Rôle du microenvironnement dans le maintien et la résistance des Cellules Souches Leucémiques.

Journées du département immunologie du CRCL (Aussois, 2013) : BMP signaling alterations in stem cells and their niche fuel tumor emergence and expansion.

Congrès du Club Hématopoïèse et Oncogenèse (Giens, 2013) : Primitive CML cell expansion relies on abnormal levels of BMPs provided by the niche and BMPRIb over-expression.

Journées de l'école doctorale BMIC (Lyon, 2013) : Primitive CML cell expansion relies on abnormal levels of BMPs provided by the niche and BMPRIb over-expression.

Société Française d'Hématologie (Paris, 2014) : L'altération de la voie BMP dans les cellules souches leucémiques et au sein de leur niche dirige l'expansion des progéniteurs myéloïdes caractéristique de la leucémie myéloïde chronique en phase chronique.

Concours « votre thèse en 180s » (Lyon, 2014) : Rôle du microenvironnement dans la résistance des cellules souches cancéreuses. Finaliste régional.

Congrès de l'ESH, 16th Annual John Goldman Conference on Chronic Myeloid Leukemia: Biology and Therapy (Philadelphie, 2014) : Impact of BCR-ABL expression on cell stiffness in a model of immature hematopoietic human cells.

Congrès du Club Hématopoïèse et Oncogenèse (Grasse, 2014) : Impact of BCR-ABL expression on cell stiffness in a model of immature hematopoietic human cells.

Journées de l'école doctorale BMIC (Lyon, 2014) : Impact of BCR-ABL expression on cell stiffness in a model of immature hematopoietic human cells.

Posters

Journées scientifiques du CRCL (Lyon, 2011) : Role of BMP4 in adhesion and resistance of Leukemic Stem Cells.

Congrès de la Science-Académie (Lyon, 2012) : Etude des propriétés mécaniques des cellules dans la Leucémie Myéloïde Chronique.

Journées scientifiques du CRCL (Lyon, 2012) : BMP pathway alterations drive Chronic Myeloid Leukemia progenitors amplification in chronic phase.

Journée scientifique du CLB (Lyon, 2012) : BMP pathway alterations drive Chronic Myeloid Leukemia progenitors amplification in chronic phase.

Congrès Club Hématopoïèse et Oncogenèse (Giens, 2012) : BMP pathway alterations in Chronic Myeloid Leukemia amplify immature progenitors compartments in chronic phase.

Congrès The Biology of Stem Cells (Paris, 2012) : BMP pathway alterations in Chronic Myeloid Leukemia amplify immature progenitors compartments in chronic phase.

Journées scientifiques du CRCL (Lyon, 2014) : Distinguishing normal from leukemic stem cells through their mechanical response to external stress. Prix du meilleur poster.

Publications

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Résumé

Une des principales causes d'échec dans le traitement des cancers est le développement de résistances aux drogues par les cellules tumorales. Les cellules souches cancéreuses (CSC) sont suspectées d'être responsables de ces rechutes, conduisant à la récurrence de la maladie et bien souvent au décès des patients. En clinique, il est donc nécessaire de développer des stratégies thérapeutiques capables de cibler ces CSC résistantes et aboutir à la guérison des patients. Les CSC sont régulées par un ensemble de signaux aussi bien biologiques que physiques au sein de la niche tumorale. Mon projet a pour objectif de déterminer l'implication du microenvironnement tumoral (voie de signalisation BMP et contraintes mécaniques) dans le maintien et la résistance des cellules souches leucémiques (CSLs) de la leucémie myéloïde chronique (LMC). Pour cela, nous avons combiné tests fonctionnels et moléculaires ainsi que l'analyse de la niche tumorale sur plus de 200 échantillons de patients atteints de LMC. Nous avons ainsi démontré que l'altération de la voie BMP intrinsèque aux cellules immatures de la LMC corrompt et amplifie la réponse à BMP2 et BMP4, présents en quantités anormalement abondantes au sein de la niche tumorale. Ces résultats récemment publiés dans *Blood* nous ont amenés à évaluer le rôle de la voie BMP dans le maintien des CSLs sous traitement par les ITK. La microscopie à force atomique nous a permis de démontrer que l'expression de BCR-ABL est suffisante pour induire une augmentation de la rigidité des cellules immatures de LMC par rapport à des cellules saines. Enfin, l'utilisation d'un système de confinement cellulaire nous a permis de démontrer que le stress mécanique contrôle la prolifération des cellules leucémiques immatures en régulant l'expression de gènes mécano-sensibles comme *Twist-1*. Ces résultats pourraient expliquer comment des CSLs tirent profit des contraintes mécaniques issues de leur microenvironnement afin d'acquérir un avantage prolifératif par rapport aux cellules saines. Ultimement, nous espérons que cette approche transdisciplinaire permettra d'identifier les molécules clés de la transduction de signaux mécaniques potentiellement impliqués dans le maintien et la résistance des CSC et ainsi proposer de nouvelles cibles pour contrer ces effets.

Mots clés : *Leucémie Myéloïde Chronique, Inhibiteurs de Tyrosine Kinase, Cellules Souches Leucémiques, Bone Morphogenetic Proteins, résistance, stress mécanique, rigidité cellulaire*

Abstract

One of the main causes of treatment failure in cancers is the development of drug resistance by cancer cells. The persistence of cancer stem cells (CSCs) might explain cancer relapses as they could allow reactivation of cancer cells proliferation following therapy, leading to disease persistence and ultimately to patients' death. Clinically, it is crucial to develop therapeutic strategies able to target resistant CSCs in order to cure the patients. CSCs are controlled by a variety of biochemical and biomechanical signals from the leukemic niche. My project aims to determine the involvement of the tumor microenvironment (BMP signaling pathway and mechanical stress) in the maintenance and resistance of Leukemic Stem Cells (LSCs) in Chronic Myelogenous Leukemia (CML). For this, we combined functional and molecular assays to the analysis of tumor microenvironment on more than 200 CML patients' samples. We demonstrated that alterations of intracellular BMP signaling pathway in CP-CML primary samples corrupt and amplify the response to exogenous BMP2 and BMP4, which are abnormally abundant in the tumor microenvironment. These results, recently published in *Blood* led us to evaluate the role of the BMP pathway in LSC maintenance under TKI treatment. Atomic force microscopy allowed us to demonstrate that BCR-ABL expression alone is sufficient to increases the rigidity of immature CML cells compared to healthy ones. Finally, using a unique cell confining system, we were able to demonstrate that mechanical stress controls the proliferation of immature leukemic cells by regulating the expression of mechano-sensitive genes such as *Twist-1*. These results could explain how LSCs can benefit from a mechanical stress exerted by their microenvironment to acquire a proliferative advantage over normal cells. Ultimately, we hope that this transdisciplinary approach will help to identify key molecules in the transduction of mechanical signals potentially involved in maintenance and resistance of CSCs and thus offer new targets to counter these effects.

Key words : Chronic Myelogenous Leukemia, Tyrosine Kinase Inhibitors, Leukemic Stem Cells, Bone Morphogenetic Proteins, resistance, mechanical stress, cell stiffness